

DEVELOPMENT OF THE FEMALE CONE  
OF PINUS RADIATA D.DON.

Volume I

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Material of two clones of Pinus radiata (19 and 55), harvested from a seed orchard at Kaingaroa, at intervals of 1-4 weeks was studied. The same clones and some others were harvested less frequently from Whakarewarewa.

Dissection and counts of terminal shoot components for each harvest confirmed that, as in other species of Pinus, female cones are initiated in summer.

Cones emerge from their bud scales prior to pollen shed in early spring. Pollination drops are produced by different ovules throughout the cone's receptive period. One ovule can produce a drop on more than one occasion. In a simulated drop, pollen floats through the fluid with its wings upward. Pollen treated with chloroform does not float, but still reaches ovule micropyles when applied to bagged cones. An electron microscope study of receptive ovules showed that the most active cells were the nucellus cap cells, some of which develop vacuoles and collapse.

Commencement of pollen shed varies with year. Duration at Whaka. (1972) was five weeks, but the timing of clonal receptivity varies. Receptive duration of cones varied from 3 to 13 days, depending on weather, and for bagged cones was shorter than for unbagged cones.

Highly significant year to year differences in the amount of pollen trapped were shown. Counts showed a highly significant difference between clones in the amount of pollen in ovule micropyles.

P. radiata ovules develop like other Pinus ovules, but the duration of developmental phases is longer. A band of elongated thick-walled cells connecting the ovule and the scale vascular tissue differentiates by fertilization.

Causes and frequency of ovule loss are described, along with other factors affecting seed production, (total scale number,

potential seeds, fertilization failure and embryo breakdown). v:

Clone 55 produces fewer seeds per cone than clone 19, but accommodates more archegonia and pollen. It has fewer unpollinated ovules, but more pollinated ovules abort. Year and site differences also affect seed yield per cone.

## CHAPTER I

## INTRODUCTION

## I GENERAL INTRODUCTION

Awareness of the need for conservation and replenishment of natural timber supplies is not new to this century. As far back as 1700 a diameter limit was put on timber removals in Sweden and later it became obvious that natural forests should be replanted and supplemented by managed forests. By 1900, with the growing understanding of genetics and heredity following the rediscovery of Mendel's work, people such as Opperman showed that important silvicultural characters, for example, straight growth and fine branches were heritable. (Griffith, 1949). It was then realised that the removal of the best trees for timber meant that the poorer remaining trees were the seed suppliers for the next naturally regenerating crop. So, it became general practice to seek out the outstanding trees for use as a seed supply. Even then the variability in vigour and suitability of offspring was considerable, so the idea of cultivating such trees collectively in a 'seed farm' or seed orchard where they could inter-pollinate, grew up (Bates, 1927). In such a situation, both male and female parents can be selected. The development of a grafting technique made clonal seed of physiologically mature ramets possible. They yield seeds in a shorter period of time and at more convenient heights for harvesting.

By the 1950s seed orchards were being established on a world wide scale. However, because of the value of improved seed (Zobel, 1971), the time involved in producing it and the expense of good land, necessary for seed orchards, there is world-wide interest in increasing

the seed yield per acre.

The New Zealand Forest Service collects about 6,800 Kg of Pinus radiata seed annually from established forests and private firms collect another 2,500 to 3,500 kg. This supplies about 43,000 hectares of planting and 17,000 hectares of replanting cutover forest. Seed orchards (yielding 24 kg of seed per ha) at present cover 240 ha, 33 being owned by private firms. At the moment plans allow for the establishment of a total of 360 ha in seed orchards by 1977 and as many of these will be immature it is thought that seed orchards will not supply sufficient seed for reafforestation in improved trees till after 1987, (Thulin, pers. comm.) It was hoped (Sweet and Thulin, 1969) that some means of increasing the seed yield in present seed orchards might be found.

To complement the physiological research being carried out on this problem at the Forest Research Institute, Rotorua, a morphological study of the development and timing of developmental events was desirable. A grant was made available by the New Zealand Forest Service for the present study.

## II THE MATERIAL

Most of the material was sampled from two clones, 19 and 55, which differ vegetatively. (Photographs and a comparison of visual differences are given in appendix 1.) Cones were sampled from Kaingaroa from 1968 to 1972 and from Whakarewarewa (Whaka) in 1971 and 1972. Site descriptions are given in chapters II and III. Other clones (7, 89, 274 and 372 ) were sampled to a lesser degree for pollination studies.

In considering seed production in seed orchards clonal data is most important, but it has not been used in the majority of previous studies on seed production in Pinus. Clonal studies which have been



made have not included morphological comparisons or information on seed losses due to aborted ovules.

### III. THE REASON FOR SELECTING THE TERM FEMALE CONE FROM ITS SYNONYMS

The term female cone was applied by Ferguson (1904) to the ovule and seed bearing structure of pines. Since then it has been called the ovulate, megasporangiate or seed cone and the term cone itself has been replaced by strobilus or flower. Of these all but flower are botanically correct, and this exception may be justifiable in discussions relating to the determinate sporogenous shoot of both conifers and woody angiosperms (Jackson and Sweet, 1972).

The term seed cone, if taken literally, would apply to the reproductive structure once seeds have been formed, (after fertilization). But Nekrasova (1970) defined the term cone as the female strobilus from ovuliferous scale closure onward, (though she still referred to female cone primordia). Other authors (Sarvas, 1962; Sweet and Bollmann, 1970) applied the term conelet to cones prior to fertilization. It seems that attempts to narrow the definition of a cone have resulted in some confusion. As this thesis is divided to cover the main stages of female cone development separately the stages have not been distinguished by special terminology, especially as that terminology is ill defined. The term female cone will be used here in its full sense because it is simple and accurate and has been used in the past more commonly than any of its synonyms.

### IV. CONTENTS OF THE THESIS

Previous studies of cone development in Pinus species have been, in general, limited to one of several aspects, (initiation, morphological development up to fertilization, fertilization or embryology). All

of these developmental stages will be described for P.radiata .

Consideration of factors affecting seed production success in this study of female cone development is justifiable on the grounds that seed production is the end result of cone development and is of practical importance. Morphological techniques (dissection and sectioning) elicit useful information for workers in physiological fields.

This investigation covers cone, ovule and seed development from cone initiation to seed maturity, a process which takes more than two years in pines. At each level of development possible factors affecting seed production are considered and finally a statistical study, comparing the effects of site, year and clone on seed yield is described.

## V. ARRANGEMENT

### (1) Text

Chapters are divided according to major developmental stages- initiation, pollination and seed development with a final chapter on seed production. There are two chapters on pollination because each aspect, the pollination mechanism within cones and the success of pollination in the stand, involves a different approach and gives plenty of material for discussion. Individual chapter indices are given for chapters II to VI so that the contents and organization can be seen from the subsection headings.

Literature associated with increasing seed yield in forest trees covers many conifer species and involves many approaches. Because of its extent the literature considered in this study has been limited to that on species of Pinus. Where possible results from other studies have been summarized so that the results obtained for P.radiata can be related to the situation in the genus generally. Because the literature is different for each chapter no attempt has been made to give a general

literature review. Literature relevant to the chapter is reported either at the beginning or in conjunction with the observations and results (to avoid repetition when the findings for P.radiata are the same as for other species).

## (2) Photographs

The life history of Pinus was fully described and illustrated with drawings at the beginning of the century (Ferguson, 1904) but there has not been a comprehensive series of photographs showing morphological development (normal and abnormal) since.

For convenience of reference photographs have been presented in a separate volume. They have been arranged, as nearly as possible, in chronological order but because the text considers each tissue's development separately at some points, the reference in the text does not always occur in the same order as the photographs appear. General headings are given at the beginning of a group of related photographs and an overlay system of labelling has been used so that low power cell and tissue details are not obliterated. Because of the sequence, the inclusion of low power photographs and the labelling, it should be possible to see ovule and seed development without using the text. It is hoped that this volume could be used by a nonmorphologist to establish the stage of approximate ovule development in the Rotorua district at any date without having to process new slides. However, it should be remembered that for some different clones, years and sites differences in developmental timing may be expected.

Except for electron microscope photographs, micrographs magnified more than a hundred times were taken on a Leitz microscope and camera unit with automatic exposure. Low power photographs 7 to 10 were taken by putting the slides in an enlarger and exposing them directly on to film, then contact printing. The remaining low power micrographs were taken by Mr F.E.McGregor using a Zeiss Ultraphot II

and most of the negatives were contact printed. All photographs, except the coloured photographs, were printed by the author.

Figures and appendices are also included in volume II so that they can be referred to beside the text. The figures are numbered according to the chapter and the order in which they appear in it. The appendices are numbered in the order they are referred to in the text.

## VI. ABBREVIATIONS

Some abbreviations have been used for the concise labelling of photographs, figures and appendices.

d.f.	= degrees of freedom
ER	= endoplasmic reticulum in electron micrographs
F.A.A.	= formalin, alcohol and acetic acid
K	= Kaingaroa
LS	= longitudinal section
meg. membrane	= megaspore membrane
m.s.	= mean square
NS	= not significant
ov.	= ovule
P.A.S.	= periodic acid- Schiff's reagent
Poll'd ('n)	= pollinated, pollination
S.E.M.	= scanning electron microscope
Sign.	= significance ..... * = significant at 5% level
	** = significant at 1% level
	*** = significant at 0.1% level
s.s.	= sum of squares
T.E.M.	= transmission electron microscope
TS	= transverse section
W	= Whakarewarewa (Whaka) Note: Longmile (figure 4:10) is an area at Whaka and has been called Whaka elsewhere in the thesis.

## CHAPTER II

THE MORPHOGENESIS OF THE FEMALE CONE AND ITS DEVELOPMENT UP  
TO THE TIME OF POLLINATION

## I. INTRODUCTION

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4. Organization of the Pine Shoot
5. The Polycyclic Shoot
6. Growth of the Pine Shoot
7. Microscopic Appearance of the Pine Shoot Apex during  
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8. The Time of Initiation of Cone Primordia
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10. Summary

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Primordia Initiation

## CHAPTER II

THE MORPHOGENESIS OF THE FEMALE CONE AND ITS DEVELOPMENT UP  
TO THE TIME OF POLLINATION

## I. INTRODUCTION

(1) General Introduction

Cone initiation is the first developmental stage when seed production in pines might be increased by alteration of environmental variables. To treat trees experimentally at this time it is necessary to know the time of cone initiation and to be able to recognise cone primordia from their position and appearance. Therefore, the bulk of this chapter will be concerned with cone initiation in Pinus radiata.

(2) Definition of Cone Initiation

Most publications on the life history of Pinus species (Ferguson, 1904; Sethi, 1928; Thomas, 1951; Konar, 1958, 1960, Konar and Ramchandani, 1962 and Nekrasova, 1970) make either a brief mention of the time of cone initiation or none at all. Despite the relevance of initiation to a study of cone development, it was research on increasing cone production in seed orchards which stimulated investigations into cone initiation. (Mergen and Koerting, 1957; Duff and Nolan, 1958). This research made it clear that cones are first formed as axillary primordia in a series of primordial buds formed by the apical meristem of certain shoots in a mature pine tree. These shoots are usually vigorous primary branches in the upper part of the crown (Waring, 1958; Hard, 1964; Owens, 1970 and many others).

To determine the time that cone primordia arose at their meristems, the terminal buds of suitable shoots were sampled at intervals throughout the year (Mergen and Koerting, 1957; Duff and Nolan, 1958). These showed that the first evidence of female cone primordia was in the summer for P.elliottii and prior to early autumn

for P.resinosa in their respective localities. Nekrasova (1970) also noted that the primordia of female cones of P.siberica in the foothills of the Urals, U.S.S.R. were established in the summer.

Ferguson (1904) could not recognise cones of P.strobus until spring but Sethi (1928) stated that the female cones are initiated in midwinter for P.roxburghii at Lahore. <sup>and Ramchandani, Konar</sup> Konar (1958) (1960), while not giving a date for cone initiation in P.roxburghii, assert that the female cones of P.wallichiana from three sites in India are initiated in the late winter.

Since these authors give no description of the appearance of female cones at the time they judge initiation, their dates of initiation are of little value, because they might really be the dates of later stages of cone differentiation.

Thus, an understanding of the components of a cone bearing shoot, their arrangement and characteristic differentiation from primordia, is essential for the recognition of cones at initiation.

### (3) Structures of the Pine Shoot

In all pine shoots studied the same basic structures and organization have been shown, but a variety of terms have been used to describe them (Duff and Nolan, 1958; Hanawa, 1966; Owston, 1968 and Owens, 1973).

Five kinds of spirally arranged structures arising from the shoot have been reported. They are cataphylls (synonomously scale leaves or bracts), needle fascioles (short shoots, dwarf branches or brachyblasts), branches (long shoots) and male and female cones. Of these, the first three are present in all pine shoots, but male cones are usually borne only on small secondary branches and very few shoots bear both male and female cones (Duff and Nolan, 1958; Curtis and Popham, 1972). Cataphylls subtend all of the other shoot structures, but, in addition, they occur in zones where they have no axillary



structures. Such cataphylls have been consistently called sterile cataphylls and, although this term is not accurate, it is concise. Nevertheless, inverted commas will be used here to mitigate the sexual connotations of 'sterile'. Cataphyll itself was selected from its synonyms because it reduces the chance of confusion with bract and ovuliferous scales of the cones.

The term needle fascicle (primordia, buds or shoots) will be used because it is accurate, descriptive and less ambiguous than dwarf or short shoot. Doak (1935) accepted the terms short and long shoot and they have since been widely used. Their rejection must therefore be justified. Doak considered that female cones were homologous to branches (i.e. were long shoots) because both arise in a comparable position and at much the same time and because cones occasionally proliferate at their tips. However, such proliferation is exceptional; normally the apical meristems of cones cease growth at a fixed stage following primordial differentiation. If cones are long shoots, but are determinate, the term short shoot with its implication (in Pinus) of a determinate structure could be confusing. If the term short shoot is rejected then the antithesis, long shoot should be also. But the newly initiated cone and branch primordia which are homologous and which can be distinguished from needle fascicle primordia, are, in some species, indistinguishable from one/another (Owston, 1968). It is therefore convenient to use the established term long shoot to apply to primordia which could develop into cones or branches.

#### (4) Organization of the Pine Shoot

Studies of pine shoot organization show that the lateral components arise in a particular order that is repeated along the shoot, and which is a reflection of the order of compact structures enclosed by cataphylls in the overwintering branch terminal bud. One of these series will be referred to as a cycle.

Bannister (1962) gives a traced map of a length of P. radiata shoot showing 'sterile' cataphyll scars at the base of an annual shoot and a zone of 31 'sterile' cataphylls above them. This is followed by a zone of 136 needle fascicle shoots. On seven of the eight parastichies above them there are cones with a branch bud on the eighth parastichy. One branch bud and three branches occur in the level above the cones, completing the cycle. A terminal cycle is not shown. The diagram of the overwintering branch terminal bud given by Curtis and Popham (1972) for P. banksiana shows the same order in each of three cycles repeated along the shoot, except that the basal cycle contains male cones and no branches. The authors note that cycles with male cones occur only very rarely in the same shoot as female cones. It is the upper part of the diagram which is of particular importance to this discussion, for, in addition to the information in Bannister's diagram, it shows that the apical meristem is enclosed in 'sterile' cataphylls and that, in P. banksiana, the uppermost (last formed) cones are not accompanied by branches.

In both diagrams the female cones that are associated with branches occur just below them in the cycle. Owens (pers. comm.) considers that this is true of P. contorta and P. monticola also. Other authors, however, have recorded that, in the species they studied, female cones occur above the branches (Doak, 1935; Duff and Nolan, 1958; Eggler, 1961 and Hanawa, 1966). From these variations it appears that, although cones and branches are associated in the shoot cycle, their precise arrangement relative to one another depends on the species. In a study of the time of cone initiation in an unfamiliar species it would be advisable to determine an expected position of cones by first looking at the position of mature cones in older cycles.

### (5) The Polycyclic Shoot

In the majority of pines one shoot cycle is formed each year as an annual growth increment. The diagrams of P. radiata and P. banksiana show that several such cycles are enclosed in the terminal bud and elongate in a single year. This has been reported also in P. palustris, P. elliotii, P. taeda and P. echinata (Eggler, 1961), P. pinaster (Debazac, 1966) and P. contorta (Van den Burg and Lanner, 1971). The term multi-nodal, used by Doak (1935) to describe the kind of growth shown by such species as P. radiata, has been discussed and rejected by Bannister (1962). But Bannister offered no alternative term. Multiple phase (Eggler, 1961) and multiple flushing (Owston, 1968) have also been used but the term polycyclic (Debazac, 1966) is preferred here because it suggests the morphological cycles rather than the elongation steps which occur when the internodes within one cycle elongate.

### (6) Growth of the Pine Shoot

Determination of initiation times of primordia at the apical meristem requires, not only an understanding of their position in the branch terminal bud, but also a knowledge of the kinds of shoot activity which occur and the time of year when they occur. In all pine species studied the shoot has been shown to undergo a period of primordial production in the summer, a rest period which coincides with the winter and a period in late winter and spring in which internodes between the shoot structures in the closed bud elongate. Elongation of the internodes separates the cataphylls which overlap to enclose the winter bud, and so the new shoot extension is produced. (Sacher, 1954; Duff and Nolan, 1958; Gifford and Mirov, 1960). In some species extension growth and cataphyll production are shown to overlap (Hanawa, 1966; Owston, 1968; Sucoff, 1971). Owens (1970) notes that cataphyll initiation occurs in early spring in P. contorta, but in P. monticola it starts about a month later and finishes about the same time in early autumn. Van den Burg and

Lanner (1971) showed that needle fascicle primordia initiation was occurring even when extension growth was in progress in P. contorta and they suggest that this earliness might be related to the polycyclic nature of its shoots. In P. banksiana new cataphyll production begins in the bud before elongation begins (Curtis and Popham, 1972). Allen and Scarborough (1970) showed that activity in the bud tip of P. palustris saplings takes place at the same time as the latter half of elongation. By this activity it is assumed that the authors mean cataphyll and primordia production, but the phrase is unsatisfactorily nebulous. However, from studies of these three species, the trend does seem to be for polycyclic species to overlap primordia production and extension growth to a greater degree than do monocyclic species. This may be a result either of extension growth covering a longer period, since several cycles are elongating, or of several cycles requiring a longer time to be initiated- or it may be a combination of both effects. However, Van den Burg and Lanner (1971) provided no reason for their suggestion of a relationship between polycyclic growth and the overlapping of new cataphyll initiation with shoot elongation. Overall, then, the time of onset and the duration of growth phases in shoots from different species and within species from different localities varies as a result of environmental and genetic differences. This was shown in an extreme way by P. radiata in Hawaii (Lanner, 1966<sup>a</sup>). Here, under tropical conditions, trees grow episodically and are not all dormant at the same time of year. Nor is cone receptivity and pollen shed restricted to a specific season. Furthermore, a proportion of P. radiata trees at Hawaii show 'foxtail' growth, where a continuous long leader, (5.6m in one case) is produced without the usual cycles marked by lateral branches and 'sterile' cataphylls. Yet twentyfour other species of Pinus examined in the same region show a normal cyclic pattern of growth and normal shoot arrangement.

(7) Microscopic Appearance of the Pine Shoot Apex during  
primordia initiation

The phase of growth which is of particular importance to this study is that of primordia production which includes the initiation of cone primordia. At the microscopic level the volume of the apical meristem can be shown to increase with the onset of its productive activity (Johnson, 1951). For this reason some authors give height to diameter ratios as a description of the apex at each stage (Duff and Nolan, 1958; Hanawa, 1966; Owston, 1969). However, Tepper (1963) found that the shoot diameter of dormant P. ponderosa apices decreased in lower branches in the tree, giving a more pointed meristem with a different H:D ratio. Such a measure must therefore be supplemented by information on position of the shoot in the tree if it is to be useful for comparisons.

Despite this volume change, cytological zonation of apical meristems in all Pinus species examined remains throughout the year the same, although interpretations of relationships to other conifer meristem zonations are variable (Sacher, 1954; Owston, 1969; Curtis and Popham, 1972). The tip of the meristem is covered by a zone of apical initials, while the more elongated cells along the sides make up the peripheral zone (or flanking zone). These surround a central zone (central mother cell region) above the rib meristem which gives rise to the pith. This zonation can also be seen in the lateral meristems of needle fascicle primordia (Gabilo and Lloyd Mogensen, 1973) and in cone and branch primordia.

(8) The Time of Initiation of Cone Primordia

In monocyclic pines in which the cones are either the last initiated structures, or almost so, the time of cone production must coincide with the end of the primordia production phase in summer or late summer. It has been mentioned earlier that the first evidence of

cone primordia in P.resinosa was around the end of summer (Duff and Nolan,1958). Cone primordia have been reported as being initiated in summer in other species also (Doak,1935, Wareing,1958, Goo,1961, Hanawa, 1966).

In polycyclic species, however, cone primordia must be formed at two or more intervals during primordia production, but there is no description of this in the literature. Egglar (1961) writes about female cones on polycyclic branches but does not describe the positions of the cones or mention the presence of cones in more than one cycle. He considers that his observations indicate that the season for cone initiation is longer than was suggested in earlier publications and thinks that female cone initiation occurs from early July to the end of August (through summer in Louisiana and Mississippi, U.S.A.) for P.palustris and P.elliottii. His observations appear to be based on limited material and are not very precise. Mergen and Koerting (1957) put cone initiation in summer but made no mention of there being more than one cycle of cones and branches in P.elliottii at Florida. Owens (1970) notes that the needle fascicle zone of P.contorta may be interrupted by branch or cone buds which suggests that cone initiation may occur earlier in the summer in P.contorta as well as occurring at the end of primordia production.

#### (9) Cone Differentiation in Pines

Following initiation primordia enlargement and cone differentiation show two distinct patterns in Pinus (Owens,1973). In hard pines (subgenus Diploxylon) the cone differentiates and continues to grow over the winter (Mergen and Koerting,1957; Duff and Nolan,1958, Gifford and Mirov,1960), but in soft pines (subgenus Haploxylon) the cones do not become recognisable until a few weeks before pollination (Owston,1969; Nekrasova,1970). This means that soft pines must differentiate the same structures more rapidly. In P.strobus, a soft pine,

from the first appearance of the ovule as a bulge in the ovuliferous scale to the formation of the integument and full organization for reception of pollen grains, only a week had elapsed (Ferguson, 1904). In P.elliottii (a hard pine) the time from the first appearance of the ovule to receptivity to pollen was about three weeks (Mergen and Koerting, 1957).

The sequence of differentiation in pines is the same regardless of the speed of development. It follows the pattern in the photographic series given by Mergen and Koerting (1957) and by Gifford and Mirov (1960). The first sign of differentiation is the cessation of cone bud cataphyll production and the enlargement of the apex of the cone into a more globular structure on a stalk. This is followed by the proliferation of bract primordia over the surface of the young cone. They are seen as raised groups of cells, which gradually increase in height. At the same time the axis of the cone differentiates a pith and the whole structure increases in size. As development continues the bract scales curve upward and extend around the cone. Then, in the axils of the upper 50% or so of the bract scales, primordia of ovuliferous scales arise and these, too, enlarge. Later (Ferguson, 1904) small groups of cells at the base of the ovuliferous scales give rise to ovules with an integument extending into a tube flared into two arms. The integument surrounds the nucellus which protects the single megaspore mother cell. At this stage cone growth pushes the bud cataphylls apart and, as the cone axis elongates, the cone scale complexes are separated, resulting in a structure which is receptive to pollen. In three species reported from different regions, cone initiation had occurred by the beginning of autumn and pollination was in spring, six to seven months later (Duff and Nolan, 1958; Gifford and Mirov, 1960; Curtis and Popham, 1972). In P.elliottii at Florida development from initiation at the end of summer to pollination near the end of winter took only five months. The authors point out that climatic and site conditions within the

natural range of this species are variable and that the time of initiation can also fluctuate from year to year. Dates given for other species at other localities show that in the northern hemisphere pollination occurs most commonly in spring or early summer (May or June). In the few records from the southern hemisphere, pollination occurs in the late winter and spring (July to September) (Millet, 1944, Fielding, 1957, Pattinson, Burley and Geary, 1967).

#### (10) Summary

In general, then, the structure of the pine shoot, its periodicity of growth and the origin and development of the female cone show only small variations among Pinus species. The timing of changes in the shoot does vary within and between species at different localities. For this reason determination of the time of initiation of cone primordia in P. radiata requires a study of both the shoot structures and shoot growth, particularly during the period of primordia production. Furthermore, determination of the time of origin of cone primordia in different cycles on the same shoot is of particular interest since it has not been described clearly for any polycyclic species.

## II. MATERIAL AND METHODS

### (1) The Material

The material for a developmental study of female cone development in clones 19 and 55 of P. radiata at Kaingaroa was collected by the New Zealand Forest Service, Forest Research Institute during the period 1968 to 1971, at intervals of either a month or a fortnight. The time between collections depended on the rapidity of morphological change in the cones. Collections were taken from grafted ramets planted in 1957 and 1958 when the grafts were 1 to 2 years old. Clones 19 and 55 were chosen because they represented the clones in



this seed orchard and yet were rather different in their vegetative appearance. (Appendix 1 contains photographs of a graft from each clone and provides a subjective visual comparison of the differences between the clones.) The seed orchard, RA1, is situated at Kaingaroa at an altitude of 549m above sea level and has an evenly distributed mean rainfall of 1473mm and mean annual temperature of 10.2°C (Sweet and Thulin, 1969).

Collections for the study of cone morphogenesis included terminal buds from primary branches in the upper parts of the crown.

## (2) Preparation for Microscopic Study

All material was fixed in F.A.A. (Formalin- 50% or 70%: ethanol: acetic acid in proportion 1:18:1 ) It was initially put under vacuum for several hours to ensure rapid infiltration into the cells. The material used in microscope sections was dehydrated in tertiary butyl alcohol in solutions of increasing concentration. It was embedded in paraplast, sectioned at 10 micrometers thickness on a rotary microtome and stained with safranin and fast green.

## (3) Determining the Number of Shoot Structures

Counts of the number of structures at the shoot tip in samples from consecutive harvests can show when new structures are being added, or have stopped being added, at the apical meristem. Fixed terminal buds were dissected by removing the cataphylls with tweezers and exposing the apical meristem, primordia and buds. The detached cataphylls were grouped for counting. Such complete dissection, (see right hand shoot, photograph 4E), was time consuming so was used mainly to determine when an increase in the number of cataphylls was occurring at the apical meristem, since at this stage they are small and closely packed around the meristem. By the time primordia initiation was completed the lower cataphylls and their axillary primordia were larger and occupied more space along the axis of the bud, so that they could

be counted along the cut surface of a longitudinally bisected bud. As the structures of the shoot are arranged spirally each one was cut at a different point relative to the others, so that in some only the outer edge of the structure could be seen, while in others the structure was cut through the median axis. Consequently, there was subjectivity in choosing which structures to count along the cut edge of the bud and the result was an estimate. In this study all the axillary primordia which could be seen along the edge were counted and in the 'sterile' cataphyll zone only the thinnest cataphyll edges were excluded. Separate counts were made on each side of the cut shoot and these were averaged.

#### (4) Limitations of the Material

To study shoot structure and growth shoots needed to be sampled all year round, and since the bulk of material which could be processed and observed was limited, the sample size of each harvest was small (1 to 5). Because estimates of the number of shoot structures ('sterile' cataphylls and axillary buds) were made and the results were variable within harvests owing to a lack of information on the number of shoot cycles below the point of harvest of each shoot, a statistical study of variation in the estimated numbers of each type of shoot structure was not justifiable. Instead, the mean number of 'sterile' cataphylls and axillary buds has been drawn as a composite shoot representing each harvest, in order to illustrate the changes in the shoots between each harvest, ( see figures 2:1 to 2:3). From these the approximate times of cone and branch primordia production can be determined. But, because precise dates cannot be given, the exact time between primordia initiation and cone receptivity cannot be calculated.

The lack of information on the number of shoot cycles in some shoots came about because the first and possibly other cycles were not harvested once elongation had occurred. This loss, associated with

the fact that at some sites P.radiata is reported as producing extension growth throughout the year (Burdon, pers. comm., 1970) means that there is no certainty as to how many cycles from the current year were excluded from the harvest. An unfortunate gap in the quantitative data results, so early cycles are not shown in figure 2:1 and are estimated in figures 2:2 and 2:3. Another difficulty arises because primary branch terminal buds on one graft differ in vigor, diameter and length, depending on their position (aspect and height) on the graft. Ideally, the primary branch buds should have been matched with one another before the harvesting schedule was started.

A further disadvantage arising from the use of fixed material was that the time when elongation of each cycle was completed had to be estimated by eye according to the distance apart of the needle fascicle buds compared to the distance in the equivalent cycle of later harvests. In a similar study it would be preferable to determine the time that elongation ceases by measurement of the growing shoots.

### III. OBSERVATIONS AND RESULTS

#### (1) Arrangement of the Shoot Structures

The structures can be seen in the photographs of terminal buds which have had their cataphylls plucked away (photographs 1 to 6). Primordia and differentiating axillary buds can also be seen in photographs of sections ( photographs 7 to 10 ). The shoot arrangement shows no deviation from the description of the P.radiata shoot given by Bannister (1962).

#### (2) Cytological Zonation of the Apical Meristem

Cytological zonation in clones 19 and 55 of P.radiata is the same as in other pines. There is a short zone of apical cells at the tip, a peripheral zone of more closely packed cells, a central region of larger, irregular mother cells and an arc of rib meristem cells

below the mother cells (photographs 11A and B). The productive meristem is fuller and more rounded than the resting meristem and the height from a base line drawn between the two last formed primordia to the tip is greater in the productive meristem. The peripheral zone of the resting meristem is narrower between the meristem surface and the underlying cells than it is in the productive meristem. The peripheral cells of the resting meristem are more elongated and the pith is wider with cells no longer arranged in straight files.

Although the zones are not marked, they can be seen in the more pointed apical meristems of the long shoot primordia (cone or branch) in photographs 12A and B. They become more distinct when the primordium differentiates as a branch (photographs 13 A and B).

### (3) The Growth Pattern

Extension growth in clones 19 and 55 of P. radiata produces a new shoot increment and reduces the closed terminal bud to a zone of 'sterile' cataphylls surrounding the apical meristem. In clone 19 shoots dissected at the end of November (photograph 1E and F) and in clone 55 shoots dissected in mid October and mid November (photographs 5B, C and F) no bud primordia were seen in the axils of the cataphylls around the closed terminal bud. However, the time when this stage is reached is different in different shoots. Because the number of cycles in the shoots sampled at this stage is unknown, some of the shoot tips from each clone will be called late cycle shoots as they have a short terminal cycle which elongates about a month later than the terminal cycle of the other shoot tips (photographs 1A to E, 5A to C and F). The penultimate cycle of the late cycle shoots, at the time of harvest, showed the same degree of elongation and needle development as the terminal cycle of the other shoots and appeared to be equivalent to it. This suggests that the late cycle shoots have an extra cycle. When the timing of shoot growth phases is being discussed these extra cycles

will be considered separately.

Graphs of the number of cataphylls dissected from around each apical meristem in a sample of 4 to 6 shoots per harvest of clone 19 (figure 2:1) showed a marked increase in their number about mid September, 1970, for early elongated shoot tips and in mid October, 1970, for late cycle shoots. This was about the time that elongation of the terminal cycle was being completed. By the beginning of November a short zone of very small needle fascicle primordia could be seen in the terminal buds of the larger shoot tips, but not in the shoots with a small diameter, nor in those with a late cycle. In equivalent harvests clone 55 shoot tips showed no sign of needle fascicle primordia in mid November (photograph 5F), but by the third week of December all shoots (from both clones) had begun to differentiate axillary primordia (figure 2:2). There were generally more in clone 55 and in the larger shoot tips of clone 19. At harvest the shoots were cut close to the newly forming bud so by December it was not possible to tell which was a late cycle shoot and which was not. However, the newly initiated primordia were the first structures in cycle 1 of the 1971 extension growth.

While needle fascicle primordia were being added, the young needles on the previous year's terminal cycles continued to grow, and reached maturity by the beginning of January (photographs 1A to F, 2A and B, 5A to D). About this time the last formed primordia in each parastichy looked slightly larger than their lower adjacent primordia and each appeared to have a broader base and to be less pointed than the needle fascicle primordia. In the following harvest most shoots had a cone or branch primordium below the 'sterile' cataphylls and in the approximate position of the highest primordium of the previous harvest, just described. New needle fascicle primordia were being initiated just below the apical meristem.

The harvest times when cone and branch primordia were identified were: the third week in December, 1970 for clone 55, the end of January,

1971 for clone 19 and, from microscope sections (photograph 7A), in early February, 1969 for clone 19. In this shoot the uppermost primordium was broader and taller than the needle fascicle primordia below them. These differences in timing suggest that cone and branch primordia formation in P.radiata at Kaingaroa, in the first cycle alone, can occur from early summer to after mid summer with as much as five week's difference between clones in one year. Differences within a clone may be due to differences between shoots or between years.

First cycle cone and branch primordia, still undistinguishable from one another, enlarge as the second cycle 'sterile' cataphylls and needle fascicle primordia are being formed. Second cycle cone and branch primordia, when they are present, arise about six weeks after the first cycle long shoot primordia (in early autumn). This is in mid February for clone 55 and from early to mid March for clone 19 (figure 2:2, photographs 3C and 7B). When there are cones in the second cycle a third cycle of shoot components is also produced, since the terminal cycle of a P.radiata shoot normally does not bear cones.

With the completion of the terminal cycle, branch buds are formed (photograph 8B). These are larger than the needle fascicle primordia below them (table 2:1, May data). Thus, the resting stage in a three cycle shoot is reached before winter (June). Vertical counts of primordia show that, in small samples of clone 55 shoots harvested from autumn (April) to winter (July) there was no observable increase in the number of axillary primordia in the terminal cycle (figure 2:3), nor did the number of 'sterile' cataphylls around the apical meristem increase after the beginning of April, so it seems that the apical meristem in these shoots had ceased producing shoot structures by this time.

Long shoot meristem							Needle fascicle meristem			
A	B	C	D	E	F	G	D	E	F	G
Feb.	19	1	2	0.37	0.24	0.31	2	0.26	0.24	0.24
March	19	1	4	0.50	0.28	0.66	4	0.31	0.24	0.24
	55	1	6	0.54	0.32	1.21	4	0.34	0.22	0.66
							+7	0.30	0.13	1.33
April	19	1	++ 8	0.44	0.22	2.20	3	0.35	0.15	0.55
May	19	1	long shoots lost				6	needle primordia		
		2	4	0.48	0.24	0.66	3	0.37	0.29	0.48
June	19	1	+++6	1.50	1.50	3.20	6	0.28	0.24	0.99
		2	2	0.37	0.22	0.40	3	0.26	0.20	0.42
							+++3	0.29	0.18	0.40

A= date of harvest; B= clone; C= cycle number; D= number of cataphyll pairs around bud; E= Width across meristem base; F= meristem height; G= bud length. + = oldest needle fascicle bud in cycle (first formed).  
 ++ = branch bud with needle fascicle buds in 4 cataphyll axils  
 +++ = differentiated cone bud  
 ++++ = needle fascicle primordium of branch bud in first cycle.

Table 2:1 Meristem dimensions(mm) in buds from one shoot in each harvest. Unless otherwise indicated the data is from the nearest needle fascicle bud below the corresponding long shoot, branch or cone bud measured. Measurements are for individual buds.

#### (4) The Number of Structures in Terminal Cycles

Since every cycle is completed by a zone of branches and possibly cones, the number of structures produced below them could affect the the time of initiation of cones and branches. Thus cone initiation might not occur simultaneously in all shoots on one tree or graft.

If it did occur simultaneously, the time of commencement or rate of primordial production must be different between shoots. It has been shown that primordia production is not evident in late cycle shoots when it has commenced in other shoots (figure 2:1) but we do not know how this affects cone production or how much variation there is in the commencement of primordia production in shoots with the same number of cycles. The final number of structures produced in any cycle is probably affected by a combination of environmental conditions prior to and during primordia production and by the internal nutrient status of the shoot. Their study is another research problem associated with cone production in Pinus. However, the number of structures counted in shoots supplied for this study will be given here to provide a record.

Up to the time that 'sterile' cataphyll increase was shown at the shoot tip, shoots from several harvests were treated as a single sample, but the sample was subdivided into late cycle shoots and other shoots. Terminal cycles of late cycle shoots in the sample always contained fewer axillary primordia and fewer 'sterile' cataphylls at the apical meristem. The mean number of all the needle fascicle primordia in fourteen late cycle shoots of clone 19 was 71.9 (S.E. = 21.1) while the mean number for the terminal cycle of other shoots was 127.4 (S.E. = 27.3). A count of needle fascicle primordia in the penultimate cycle of fourteen late cycle shoots gave a mean of 143.3 (S.E. = 22.7). Analysis of variance showed that there was no significant difference between the penultimate cycle of a late cycle shoot and the terminal cycle of other shoots, but there was a significant difference between the penultimate cycle of a late cycle shoot and the terminal cycle of ~~these~~ shoots, (1% level).

Bannister's (1962) count of 31 'sterile' cataphylls and 91 needle fascicle shoots in the terminal cycle of the twenty sixth annual shoot of a P. radiata tree is comparable to two counts from late cycle shoots sampled here. (33 'sterile' cataphylls to 93 needle fascicle shoots and



25:90). However, in this clone, at this site, there were generally fewer needle fascicle shoots. The mean number of 'sterile' cataphylls in the terminal cycle of late cycle shoots was 32.5 (S.E. = 7.7).

#### (5) Growth of Shoot Structures in the Closed Terminal bud

As the formation of structures progressed through summer and early autumn, the earliest formed needle primordia became larger and differentiated their own bud cataphylls. At the beginning of autumn (early March) needle fascicle primordia in the first cycle had developed ~~three~~ needle primordia, a group of regular cylindrical mounds replacing the meristem (photograph 9C and D). But, primordia in the terminal cycle had not differentiated by the beginning of winter (early June) (photographs 8A and 8B).

First cycle cone and branch primordia enlarge and gradually emerge as buds from the scales of the terminal bud (photographs 3A,B and C). Cone differentiation begins when cone primordia cease to produce cataphylls. Branch buds continue to produce cataphylls and become larger than cone buds. About a month after the estimated time of primordia initiation the lowermost buds among the longshoot primordia look smaller and have fewer cataphylls and a paler apex. First cycle branch buds begin to form their own needle fascicle primordia (photograph 3D) before mid autumn and continue to produce them. A microscopic section is shown in photograph 8C. By mid autumn dissected cone buds have a stalk, bearing cataphylls which surround the smooth, white globular cone meristem (photographs 3E and 4D). Second cycle cone and branch primordia reach a similar stage by the end of autumn (photograph 3E).

#### (6) Growth and Development of Young Cones

Cone development during the winter is like that shown for other species of hard pine (Mergen and Koerting, 1957; Gifford and Mirov, 1960). The smooth cone meristem develops a surface pattern of small 'bumps' which

are the bract scale primordia (photograph 8D). These can be seen at the beginning of winter in first cycle cones when the cone is about 3.2mm in length (from the apex to basal cataphylls) and 1.6mm across at its widest point. Enlargement of the bract scales so their bases meet gives a geometrical pattern to the surface of the cone. This can be seen about three weeks after the bract scale primordia appear, during which time the cone enlarges further. The cone measured was 5mm by 2.3mm. In section the bract scales point upward around the cone and develop ovuliferous scale primordia in their axils before mid winter (photograph 10A). These grow over the next six weeks and the upper half give rise to two groups of cells at their base. Each group becomes differentiated into three lobes of the ovule; the larger middle lobe is the growing nucellus and the outer two lobes elongate to form integument arms (photograph 10D). This occurs in the late winter or at the beginning of spring, (August to early September at Kaingaroa).

The lowermost ovuliferous scales of the cone do not bear functional ovules, although those near the middle of the cone may have flaps of rudimentary ovule-like tissue at their bases. The number of ovule bearing to non ovule bearing scales in cones of P. radiata and the appearance of rudimentary ovules will be described in chapter VI.

Shortly after the ovules have differentiated the cone axis elongates and the cone emerges from its bud scales. The stages of cone development from emergence to receptivity are described in chapter III.

Second cycle cones generally become receptive only a week or so after the first cycle cones, which means that their time for development from primordia to pollination is shorter. Clone 55 cone primordia (some of the long shoots) were present on 21-12-70 and pollination was about 20-8-71 (about eight months later). Clone 19 first cycle cone primordia were present about 20-1-71 and the cones were probably pollinated about a week after those of clone 55. This puts the developmental

time for clone 19 cones at just over seven months.

A comparison of photographs 10B and 10C shows that the smaller, less developed cone in 10B was harvested in 1969 ten days later in the year than 10C, harvested in 1968. Field observation also shows that cones on one tree during the pollination period may be at different stages of receptivity so it is not possible to say if the difference shown here is due to within clone or between year variation. It is possible that both kinds of variation occur. A more detailed description of variation in times of emergence and receptivity in P. radiata will be given in chapter III.

#### IV. DISCUSSION

##### (1) Shoot Structure and Growth

P. radiata, clones 19 and 55 shoots, at Kaingaroa, showed the arrangement of structures seen in other species of Pinus. Their growth phases- spring elongation, summer primordia production and winter rest- with some exceptions, correspond to those of other species. However, at this site the period of winter rest is brief as elongation of the first, and sometimes the second cycle internodes, occurs in winter prior to pollination. Although the estimation of elongation of the terminal cycle was subjective, P. radiata here is unlike the other polycyclic pines in which spring elongation and cataphyll production overlap to a marked degree. On the other hand, P. palustris in Southern Mississippi (Allan and Scarbrough, 1970) also begins elongation in mid winter, but it shows cataphyll production in conjunction with spring elongation of the terminal cycle. P. radiata differs from other species in that it may undergo a short summer-autumn elongation of the internodes between the 'sterile' cataphylls at the base of the new primary shoot terminal bud.

## (2) Recognition of Cone and Branch Primordia

During summer cone or branch primordia (long shoots) can be distinguished from the needle fascicle primordia below them by their greater volume. The difference in size can be measured from longitudinal sections of the primary shoot terminal bud, but can also be discerned by eye, when the cataphylls are plucked from the bud tip.

## (3) The Time when Cone and Branch Primordia Production Occurs

P. radiata shoots examined from Kaingaroa, like those of P. elliotii, P. echinata and P. palustris (Eggler, 1961), initiated cone and branch primordia at any time between early and late summer, depending on the clone and on the particular shoot cycle. The polycyclic nature of the shoot does not alone account for the extended period of cone and branch primordia initiation. In equivalent cycles the time of origin of cone and branch primordia in different clones can differ by up to a month, possibly longer. Careful examination of clonal material of other species of Pinus, at one site, might also show a longer period of cone primordia production in monocyclic species, than is implied by single dates given in the literature.

However, in P. radiata the period of cone and branch primordia production is extended further by the formation of second cycle cone and branch primordia, about six weeks later. Thus, the period between the production of the earliest first cycle cone primordia and the latest second cycle cone primordia could be greater than two and a half months. Third cycle (and very rarely fourth cycle) cones seem to occur on the leader shoots of 'early' initiating clones. Therefore, if about six weeks elapses while a whole cycle of shoot structures are being initiated, it seems unlikely that the period during which the great majority of cone primordia are being initiated is greater than three months. Furthermore, the counts of the number of structures in each cycle (figure 2.1) show that later formed cycles have fewer structures.

It may, then, take less than six weeks for the completion of a later cycle. Also, the rate of production could be greater in vigorous leader shoots, so the period over which cone primordia are initiated could be shorter than three months.

#### (4) Differentiation and Development of Cone Primordia

Because the number of cones differentiated affects the final cone crop, the time when cone primordia become different from branch primordia is of significance to research aimed at increasing cone production. A literature of initiation and differentiation is reviewed by Sweet, 1974. Hormonal or nutrient treatment intended to increase the number of cone primordia formed might also increase the number of branches formed, if applied at the time of primordia initiation. But, if it were applied at the time of cone differentiation, the proportion of cones to branches might be increased.

Cone and branch primordia become distinguishable from one another about five weeks after the time of initiation. When the terminal bud cataphylls are removed, the lower buds in the long shoot zone look smaller and not as cylindrical as the buds above them. When dissected the lower buds do not have as many bud cataphylls. This size difference in structures which must have been initiated at virtually the same time, means that differences in growth rate must have occurred well before the time at which the structures can be distinguished visually. Furthermore, from the correlation of difference with position and the numbers of each bud type, it is suggested that the upper primordia of the long shoot zone are destined to be branches and the lower ones to be cones from a time quite close to primordia production.

Owens (1973) suggested that all primordia in the cone-branch region in P. monticola are potentially able to become either cones or branches. If this is true, under extremely favourable cone bearing

conditions one would expect that all such primordia could become cones and under alternative extreme conditions all the primordia might become branches. This latter situation is normal in non primary and low branches. Although it is rare for pure cone regions to form on a shoot it is possible that favourable conditions for cone production in the shoot could enhance the proportion of cones produced in the cone-branch zone. (Single cones, unaccompanied by branches occasionally occur in second cycles of small diameter branches near the top of the tree.) The required conditions to enhance cone production could be at or shortly after the long shoots are initiated, even though the cone and branch primordia are not visibly different at this time.

Following the cessation of bud cataphyll production by cone primordia, a change in the apical meristem occurs and it becomes globular. Development from this time onward is the same in P. radiata as in other species described.

#### (5) Implications of an Extended Period for Cone and Branch Primordia Initiation

The time taken for cone development from primordia initiation to receptivity varies according to the time of primordia initiation. The difference in time of cone receptivity in equivalent cycles from these two clones is around a week, but the difference in initiation times is about a month. Similarly, the difference in receptivity times between successive cycles on the same shoot is about a week, although the difference between initiation times is much longer (around six weeks between first and second cycles of the same clone). In this material the time taken for cone development, from primordia initiation to receptivity is between six and eight and a half months. This is comparable to that of the few northern hemisphere species for which the developmental time can be calculated.

There is also evidence that the clones initiate their long

shoot primordia on different calendar dates each year (figure 2:2). Pollination times also vary by some weeks. However, it would require detailed data from several years to show whether pollination times are related to initiation times or to subsequent environmental conditions.

The fact that cone and branch primordia can be initiated in different primary shoots over a three month period suggests that a single external environmental variable is not the stimulus for converting needle fascicle production to cone and branch primordia production. There is evidence from the greater consistency of timing within clone samples, that a genetic factor plays some part in regulating the initiation time of long shoots and later, to some extent the time of cone receptivity. The timing of clonal receptivity will be described more fully in chapter IV. The establishment of the factors involved with cone initiation and their mode of action is a physiological problem. The application of experimental treatments relative to known dates of initiation may help to determine the physiological processes so that appropriate treatment to increase the number of cones initiated and differentiated may become possible.

The staggered time of cone primordia production in different clones and the occurrence of many second and third cycle cones, over a period of several months, would pose problems for the treatment of whole seed orchards. In a clonal seed orchard at least some shoots will be producing cone primordia at almost any time during the summer. Thus, treatment designed to enhance cone primordia production and survival, must be applied over a greater period than is necessary for individual grafts. Alternatively, treatment could be applied to specific clones at their individual optimum times. This would require experiments to determine the optimum time for treatment and a way of determining the precise stage of shoot development for the clone.

The kind of treatment suggested above would require a great deal

of preliminary research and would perhaps be complicated to carry out. It might be more practical to find the site conditions which are most conducive to cone and seed production and to establish seed orchards on such sites.



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## CHAPTER III

## POLLINATION

## I. INTRODUCTION

(1) The Importance of Pollination

Pollination is the process by which the male gametophytes of a species are transferred to the proximity of its female gametophytes. In Pinus it involves both the transport of pollen by air currents and the reception of pollen by the female cone. Following development within its bud, the female cone emerges as an effective pollen trap (Sarvas, 1962). But, once the flight of pollen has been successfully intercepted the pollen must still be transferred to the interior micropylar chamber of the ovules, where there is a sheltered environment for pollen germination.

For tree breeders who carry out artificial pollination an understanding of this process could lead to improved techniques and better seed yields. Furthermore, as low pollination levels have been shown to be the cause of reduced seed production in natural stands of P.sylvestris (Sarvas, 1962), knowledge of the factors affecting pollen transfer and cone receptivity could be applied to problems concerned with seed production in seed orchards.

(2) Reasons for Considering Pollination Processes in the Cone  
Before considering Pollen Shed

An understanding of the complete process of pollination requires a study of factors affecting pollen shed. However, since information on the quantity and patterns of pollen shed has been gathered from measurement of the pollen levels in cones which were receptive at known times, it is necessary first to understand the processes of pollination which occur inside the cone. Although this arrangement of material is not

chronological, it means that the implications of results from the later described studies of pollen shed can be related to the success of pollination of ovules in the cones.

### (3) Structure of Receptive Cones

Recent authors (McWilliam, 1958; Sarvas, 1962) have omitted descriptions of the structure of a pine cone, although Sarvas emphasised its suitability as a pollen trap. Because pollination is a multistep process it is necessary to have a clear idea of the spatial arrangement of cone structures.

The cone consists of an axis bearing spirally arranged scale complexes (photograph 10D). Each complex is made up of a ventral bract scale and a dorsal ovuliferous scale (photographs 16A and B). Of the latter those in the upper half of the cone bear two ovules close to the cone axis (Doyle and O'leary, 1935; Sporne, 1965). The ovule at pollination is made up of a lobe of actively dividing cells, the nucellus, surrounded by an integument which is free around the nucellus tip and encloses the micropyle or pollen chamber (photographs 16D and 17C). The integument is extended to form the micropylar canal with two extensions, micropylar arms, projecting downward from the micropylar canal rim and over the edge of the ovuliferous scale (photographs 16A to D). At the chalazal end of the nucellus, (the end away from the micropyle), embedded in the nucellus centre, is the megaspore mother cell, surrounded by several layers of small, dense cells, the spongy cells (Ferguson, 1904) (photograph 17C).

### (4) Arrival of Pollen in the Cone

When the cone is receptive wind borne pollen is carried between the cone scales. The micropylar arms, which appear to be sticky, intercept pollen because they hang down into the channel between the scales. The pollen adheres to the micropylar arms.

However, the observation that most of the pollen inside a cone

is concentrated around the arms and the micropylar rim led to an investigation into the possibility that a bioelectric phenomenon could operate (McWilliam, 1959). In that investigation it was shown that pollen grains in the air generally bear a negative electric charge. But, no evidence was found for an electropotential gradient which would direct pollen into the cone. It is possible that micropylar arms, which extend to fine points, can accumulate charge and, although this might be too small to attract pollen from the external air, it might exert a pull on pollen grains once they reach the cone-scale channels.

#### (5) Transference of Pollen into the Micropyle

Pollen on the micropylar arms must be transferred upward through the micropylar canal into the micropyle (Doyle and O'Leary, 1935). The pollination drop was observed in conifers in 1841 by Vaucher, and since then its behaviour has been described in detail (Tison, 1911). In Pinus it was suggested that pollen floats through the liquid of the pollination drop up into the micropyle (Doyle and O'Leary, 1935). The function of the pollen wings was thought to enhance flotation through the pollination drop. They were not necessary, as was previously thought, for facilitating pollen flight in air; larger, heavier pollen of Larix and Pseudotsuga species is wind borne and does not have wings.

However, McWilliam (1958) reorientated branches so that the cones were inverted. He control-pollinated them and found that pollen could still pass into ovules as far as the nucellus tip. From this he concluded that pollen grains do not float through the pollination drop but are drawn in by its resorption and drying. Sarvas (1962) showed that each micropyle can accommodate one or more pollen grains, but only one is necessary for an ovule to continue growth, but McWilliam has not provided data to indicate whether there was a reduction in the number of pollen grains present in the micropyles of ovules pollinated in inverted cones. If there had been such a reduction, total rejection of

the flotation mechanism would not be justifiable; some pollen could fall into the micropyle in inverted cones.

McWilliam (1958) also attempted to prevent the formation of the pollination drop by stripping needles from the shoot and enclosing the cone with a plastic bag containing silica gel crystals. In most cases the ovules became pollinated, but it could not be determined whether or not drop formation had been prevented completely. In both of these experiments McWilliam based his conclusions on small samples which gave inconsistent results.

McWilliam, too, rejected the idea that the pollen wings orientate the grains in the micropyle so that the pollen tube, which emerges from between the wing bladders (ventral surface), is directed toward the nucellus. Ferguson (1904) illustrated a pollen grain with its dorsal surface against the nucellus tip and Sarvas (1962) estimated that a quarter of the pollen grains showed this orientation. In these cases the pollen tubes emerge from the side away from the nucellus and must bend around in order to penetrate it. McWilliam (1958) rejected Doyle and O'Leary's suggestion for the function of the pollen wings without discussing alternative functions.

#### (6) Functioning of the Pollination Drop

The precise stage of cone receptivity at which pollination drop production commences has not been established. It was stated that drop production occurs in mature ovules (Doyle and O'Leary, 1935), from which it is assumed that the authors are referring to ovules in cones which have reached peak receptivity. Sarvas, 1962, however, was of the opinion that the pollination drop was produced in ovules as soon as pollen could pass between the cone scales.

Although it has been stated that pollination drop production could be repeated until pollen arrived and then the drop withdrawn almost immediately and completely (Doyle and O'Leary, 1935; McWilliam, 1958),

Sarvas (1962) considered that ovules secrete fluid over several days even if some pollen has reached the nucellus tip. The reason for this opinion was that by the time pollination is completed, all the excess pollen grains from the micropylar arms are crowded at the micropylar rim.

In an unpollinated cone the appearance and disappearance of the pollination drop was thought to be controlled by water relations within the cone and the external atmospheric humidity, as in the process of guttation (McWilliam, 1958). Drop production is generally observed at night or in rainy conditions (McWilliam, 1958; Sarvas, 1962). Sarvas pointed out that evaporation may play a part in the disappearance of the pollination drop, but if humidity were the sole regulator, simultaneous drop production by all ovules of a cone would be expected. This does not appear to happen.

In P. nigra pollination drops analysed by McWilliam (1958) were a 1.25% solution of sugars, a mixture of fructose and glucose with a smaller proportion of sucrose. According to McWilliam this is comparable to the composition of guttation fluids of maize and to the tissue fluids of pine needles. He does not say whether they are xylem or phloem tissues in pine needles. However, explanations of the guttation process are based on the phenomenon of root pressure, and, so far, root pressure has not been conclusively shown to occur in conifers (T.T.Koslowski, pers. comm., 1973). McWilliam himself remarks that the sugars in drops of P. nigra have been shown to be those present in the nectar of many flowering plants. It was shown that sugar forming the nectar of Lonicera japonica came from cells containing lamellar endoplasmic reticulum, which reached maximal development at the time of nectar secretion (Fahn and Rachmilleritz, 1970). Nectary cells in Abutilon are rich in mitochondria and endoplasmic reticulum (ER). They have small vacuoles which enlarge and coalesce during nectar production (Findlay and Mercer, 1970). In Plantago ovata (Hyde, 1970) mucilage is deposited between the plasma

membrane and the cell wall, accompanied by an increase in the number and size of the Golgi vesicles. In these cases sugar solutions may be actively secreted rather than guttated.

Since pollen of other Pinus species can reach the micropyle of an ovule (Doyle and O'Leary, 1935), the pollination drop must be a non-selective transporting mechanism. This is borne out by the presence of dust and insect eggs in some micropyles (Sarvas, 1962).

As pollen of any one tree is usually being shed when at least some of its female cones are receptive, as well as hybridization, self pollination is possible in Pinus.

#### (7) External Changes in the Female Cone over the Receptive Period

From the time a cone emerges from its bud cataphylls to the time its ovuliferous scales close the cone increases in size, in the depth of its colour, and changes in the degree of its accessibility to pollen. Despite continual gradual changes in cone development, stages of receptivity can be recognised and arranged in a developmental scale. During the pollination period cones can be assessed according to the stage of receptivity each has reached, for practical and experimental purposes. The scale devised for this study (Table 3:1) is comparable to those of Cummings and Righter (1948), Wakely and Campbell (1954), Ehrenberg and Simak (1956) and Pattinson, Burley and Geary (1967), which were used to determine the optimum stage to bag and to control-pollinate pine cones.

The stage of peak receptivity to pollen is reached when the cone is fully emerged from its bud cataphylls, when its axis has elongated so that the cone scale complexes are widely separated and when the colour begins to deepen.

#### (8) The Colour of Receptive Cones

Young cones of thirteen species of Pinus for which the colour has been described are a shade of red (from pink to purple) at the



time of their pollination. P.wallichiana has pale green cones at receptivity. (Konar, 1962).

It has been suggested that red pigments (anthocyanins) protect the cone tissues from excessive solar radiation (Krugman, 1956). Since cones are held in positions of high light intensity the idea seems reasonable. Anthocyanins of young cones in the Pinaceae have been identified (Santamour, 1966).

#### (9) Changes in Cone and Ovule Tissues following Peak Receptivity

Before pollination the integument is an undifferentiated zone of tissue containing scattered tanniniferous cells (Singh and Johri, 1972). When pollen reaches the nucellus tip the middle cells of the micropylar arms elongate across the axis of the arms and the swollen tissue closes the micropylar canal (Ferguson, 1904; McWilliam, 1958). Ferguson's diagram shows that the external tips of the micropylar arms wither beyond the point of swelling.

The concave nucellus tip is lined with irregular protruding cells (Ferguson, 1904). These are described as being elongated, thin-walled, vacuolate cells which are frequently capped by a mass of darkly stained disintegrating tissue (Pattinson et.al., 1967).

The spongy tissue undergoes mitosis and differentiates while the megaspore mother cell enlarges and its nucleus becomes more densely stained.

Growth of the ovuliferous scales throughout the pollination period is vigorous and exceeds that of the bract scales. The ovuliferous scales swell so that they eventually meet and seal the cone. This occurs at the same time that the micropylar canal becomes closed (Pattinson et. al., 1967). In some species interlocking hairs or elongated epidermal cells have been described on the surfaces where adjacent ovuliferous scales make contact (Thomas, 1951; Konar, 1960). These do not occur in P.radiata.

## II. MATERIAL AND METHODS

### (1) The Material

Observation and experiments were carried out in the springs of 1970 to 1973 on grafted clones established at Whakarewarewa in 1968. The site at latitude  $38^{\circ}$ , longitude  $176^{\circ}$  is 307m above sea level. It has a mean annual rainfall of 1500mm and a mean annual temperature of  $15^{\circ}\text{C}$ . Soil at the site is sandy loam of pumice origin (Burdon and Low, 1971).

### (2) Experimental Methods

In 1970, 1971 and 1972 artificial pollinations, designed to effect pollination (pollen arrival in the cone) at a known stage of cone receptivity, were carried out in the established way (photograph 15C). Cones were later harvested at known times and were sectioned so that pollen counts could be made.

In addition, pollen treated in one of the following ways, was applied artificially and the success of pollination determined from sections of the treated cones.

a. A sample of P. radiata pollen grains were treated by immersing them in a beaker of ethanol and then drying. They were then shaken in chloroform for two hours and redried. This apparently ruptured the pollen wings so that most of them sank in water. The non floating pollen was decanted, dried ready for application to bagged cones.

b. Pseudotsuga taxifolia pollen, collected the previous year and stored, was applied to P. radiata cones.

c. A sample of pollen grains was immersed in brown-black hair dye, which killed the pollen but did not affect its ability to float. Pollen marked in this way could be applied to unbagged cones and could be identified inside ovule micropyles by later dissection or sectioning. In dissection the black grains show up against the yellow grains which have been carried by the wind. Cones for sectioning were left until the pollen had germinated, in the grains which had arrived by natural means.

The black pollen, applied artificially did not germinate.

Pollen can also be stained with safranin and fast green (separately), but the pollen remains viable and <sup>the colour</sup> does not persist through the embedding process.

### (3) Microscopy

Traditional sectioning and staining techniques using safranin and fast green were used. At the time of pollination it is possible to section whole cones which have had the scales bearing rudimentary ovules removed, so that more sections can be fitted onto the slides. The tips of the ovuliferous scales can also be trimmed away. In serial section each ovule in the cone can be followed until its micropyle is in view. Pollen grains in the micropyle can be recorded as each new one appears.

~~As the time taken~~ to make pollen counts on sectioned material was considerable sampling was restricted to thirty ovules per cone. Burdon and Low (1971) showed that the mean number of ovules per cone in a sample of cones from ten clones at Whakarewarewa was 144, so 30 ovules would be about 20% sampling.

### (4) Dissection

In material in which the micropyle has not yet closed it is possible that some pollen grains are lost during evacuation of the cones in F.A.A. The pollen grains in the micropyles of receptive ovules could be counted from dissections and many ovules could be examined.

In dissection individual scale complexes of a freshly harvested cone were sliced from the cone axis with a needle tip. They were held at needle point on the stage of a stereoscopic microscope. An incision was made, extending from the micropylar rim along the length of the ovule for the depth of the integument, so the integument could be folded to one side, exposing the nucellus tip and the micropylar canal (photograph 17D). The pollen grains can be counted in situ and the number checked by removing them one by one. Forty ovules were dissected per cone.

(5) Preparation of Material for Electron Microscopy

a. Scanning electron microscopy.

Freshly picked cone-bearing shoot tips, wrapped in moist cotton wool and enclosed in plastic bags were taken (by air) to Department of Scientific and Industrial Research, Physics and Engineering Laboratory at Lower Hutt. They were dissected, freeze dried, coated in carbon and gold palladium and observed under the S.E.M.

b. Transmission electron microscopy.

Material to be examined for evidence in ovules of secretory cells which could produce the pollination drop, was air freighted to Christchurch. Shoots from clones not studied in other aspects were picked because they bore cones at appropriate stages of receptivity. Cone scales from cones of various stages of receptivity were fixed in 2% gluteraldehyde and 3% formalin in 0.05M phosphate buffer at pH 7.2 for four hours at 4°C. The material was evacuated for two hours and washed in phosphate buffer overnight. This was followed by treatment in 2% osmium tetroxide in phosphate buffer (0.025M, pH 7.2) at 20°C, dehydration in alcohol at 2% stages, embedding in Spurr's medium and polymerization at 70°C for eight hours. The sections were stained in lead citrate and uranyl nitrate.

Material which had been fixed in 2% potassium permanganate for two hours and dehydrated in acetone at 10% stages before being embedded in Spurr's medium was not fixed sufficiently to yield useful information.

Material for E.M. study was cut at 90μ. At intervals during cutting, thicker sections were cut at 1μ, placed on a glass slide and warmed so that they would adhere to it. They were stained in toluidine blue (appendix 5D). Such a technique is useful for showing the tissue arrangement of sections being studied under the E.M. and also provides well fixed thin sections, which show cell detail more clearly than in wax embedded specimens (photographs 17C and 18A-F).

symbol	description
b	Blunt: cone buds swelling, no longer pointed.
JE	Just emerged: Cone scale complexes (pink) appear at bud tip.
$\frac{1}{4}E$	Quarter emerged: a quarter of cone scale complexes exposed.
$\frac{1}{2}, \frac{3}{4}E$	The corresponding proportion of cone scale complexes exposed.
EFE	Early full emergence: All cone scale complexes are exposed, but they point upward and the green--pink bract scales give a delicate appearance to the cone.
ER	Early receptivity: Cone scale complexes are horizontal; the pointed tips of the light red ovuliferous scales are evident.
R	Receptivity: cone scale complexes are wide apart, showing the cone axis. The rosy, succulent looking ovuliferous scales over lap the bract scales.
LR	Late receptivity: Deep red--purple ovuliferous scales are swelling to reduce the channels between them.
EC	Early closure: ovuliferous scale tips swollen: edges of bract scales visible between the ovuliferous scales, but there are no channels between the scales.
C	Cone enlarged and dark purple; closed. Bract scales not visible.

Table 3:1 Description of recognisable stages in the development of female cones over the period of receptivity to pollen. Some of these stages are illustrated in photographs 14 A to D and 15 A and B.

### III. OBSERVATIONS AND RESULTS

#### (1) Direct Observation of Pollination Drops

Pollination drops were seen in bagged receptive cones on potted grafts which were kept under humid conditions in the glasshouse. The potted grafts could be laid along the bench so that the interior of the cones could be viewed through a stereoscopic microscope. On a wet day when the humidity in the glass house reached 90%, almost all ovules in one receptive cone had large spherical drops hanging from their micropylar arms. In other receptive cones, scattered drops were seen. Scattered drops were also seen in the same cones on subsequent days when the humidity was 60%. The cone which had produced many drops produced a few scattered drops five days later. Drops were not always full, spherical and surrounding the curved micropylar arms; often they extended only half way along the arms beyond the micropylar rim and looked like a film of fluid stretched between the two arms, as in the diagram given by Sarvas, 1962.

Drops seen under the microscope and left for several hours did not disappear even when pollen grains were flicked onto their surfaces. Usually pollen grains floated on the surface of the drop and would move around if they were blown. Many of them would come to rest against the micropylar rim, but they did not rise through the drop into the micropyle. Movement of pollen grains into the micropyle may have been hampered by the orientation of the cone (on its side).

In the field cones were harvested at intervals during one night (10 p.m., 4.30 a.m., 5.20 a.m. and 6.00 a.m.). They were examined almost immediately under a stereoscopic microscope. No drops were seen in nine prereceptive and receptive cones harvested at 10 p.m., but a few were found in the first three cones harvested at 4.30 a.m.. Although twenty eight cones were harvested after this, no more drops were seen, even though the night was humid with occasional showers. Two cones in which

drops were seen, were receptive, but the other with two drops was assessed at  $\frac{1}{2}$ E. Cones at all stages had trapped very little pollen so it appeared that pollen shed was not far advanced. Since many cones with no drops were receptive, or at LR, it seems possible that at least some of their ovules had produced drops that night. If ovules in the cones examined had produced drops, they may have done so between 10 p.m. and 4.30 a.m. and that they <sup>may have</sup> ~~were~~ <sup>been</sup> also dried or <sup>been</sup> resorbed during this period.

In very wet weather cones harvested in the field and taken to the laboratory for examination were seen to have occasional pollination drops, even though there may have been half an hour's delay between harvesting and examining. Thus, in humid conditions, drops do not disappear rapidly, either when pollen is applied or when they are subjected to mechanical shocks (picking and movement).

These observations show that a watery drop is produced in P. radiata ovules in receptive cones, but there was no evidence that the drop transports pollen into the micropyle. The presence of pollination drops in very humid conditions and their rarity in field conditions (during the day and during the period of observation one night), could suggest that the drops are an incidental phenomenon associated with high humidity. If this is so, an alternative process for pollen transport upward into the micropyle must be found, but none is evident.

Therefore, to investigate the behaviour of pollen on a drop of sugary fluid an artificial system was set up and viewed through a stereoscopic microscope. A simulated pollination drop (made up according to McWilliam's figures for the composition of a pollination drop: 33 mM glucose, 40 mM fructose and 2.5 mM sucrose solution) was sucked into a 1 ul microcapillary tube, which was clamped vertically. The diameter (0.20mm) of the tube was about 2.5 times that of a micropyle (0.08 mm). Two small hairs were attached to each side of the tube end with melted wax, immitating ovule arms. Pollen was puffed from a glass dropper onto

the hairs and grains were seen to adhere to them. When a drop was squeezed from the capillary tube the pollen on the hairs immediately moved inside the surface of the drop and floated upward, either around the perimeter of the drop or directly through the centre of the drop to the capillary mouth. Many grains floated up through the capillary tube and all had their wings uppermost. The pollen moved to the capillary mouth ( the artificial equivalent to the micropylar rim), so that for successful pollination in natural conditions the pollination drop would need to be present around the micropylar arms for only a short time. If the drop dried quickly or was actively resorped by ovule tissues, the rarity of drops in field conditions would be explained. However, it is possible that some ovules contain pollination drop fluid in the micropyle and micropylar canal (as far as the rim), which is not visible externally.

In addition, nonfloating pollen (prepared according to the method described in section III, 2a) was puffed onto the artificial ovule arms. This time when the sugar drop was squeezed out of the capillary mouth, the pollen beneath the drop surface slowly moved to the bottom of the drop and remained there. Puffing air onto the drop caused the pollen to swirl around within the drop, but it sank again.

The same differences between floating and treated pollen is shown more simply by applying treated and untreated pollen to drops of water or sugar solution on a microscope slide. Examination through a stereoscopic microscope shows that untreated pollen floats to the top, crowding at the highest point of the drop with the wings upward. Treated pollen sinks through the drop onto the surface of the slide.

Therefore, pollen can float upward through the centre or just under the surface of a drop and can float through a sugar solution in a capillary tube, providing the wings are intact. Furthermore, the wings orientate the grains so that the ventral (germination) surface is directed



upward. These points contradict McWilliam's (1958) conclusions, so more detailed study of the success of pollination with nonfloating pollen and of the orientation of the pollen grains was necessary.

Nonfloating pollen was applied to bagged cones of clone 172 in 1970. The cones were left until closure and then prepared for microscopic counts, from which it was shown that nonfloating pollen did still reach the micropyles, even though the wings often appeared misshapen and flattened. Figure 3:1 shows that the number of pollen grains accommodated in the micropyles of thirty ovules was related to the number of pollen grains trapped in the cone, and when more than 200 grains were trapped in the cone, the number of pollen grains in the micropyles averaged more than three per ovule. In section 2 it will be shown that the stage of cone development at which pollen is applied affects the amount trapped by the cone and in chapter IV it will be shown that the amount of pollen in the micropyles depends on the amount of pollen trapped - up to the point where all the micropyles in the cone are filled to capacity. The evidence, therefore, suggests that it was the amount of pollen in the cone which affected the pollination success when nonfloating pollen was applied, not the treatment of the pollen. Variation in the amount of pollen in the cones was probably due to variation in stage of development between cones at the time of pollen application. When sufficient pollen was trapped by these cones the amount reaching the micropyles was comparable to that achieved by artificial pollination with untreated pollen. (Compare pollen levels- y axis- in figure 3:1 with those for clones 222 and 398 in figure 3:8).

Artificial pollination with Pseudotsuga taxifolia pollen succeeded in pollinating twelve ovules in a hundred and fifty, taken from six cones of clone 222. Only one cone had failed to trap large amounts of P. taxifolia pollen. In this experiment the size of the pollen probably inhibited its transport, because one grain occupies about 4.4 times the

volume of a grain of Pinus radiata. The transport of such large grains which fill the micropyle completely suggests that the transporting process must be quite powerful.

These experiments support McWilliam's conclusions from inverting cones, that pollen can get into ovule micropyles when the flotation mechanism is counteracted. This suggests that another mechanism must withdraw nonfloating pollen (and occasional dust particles) into the micropyle. Withdrawal could occur as the pollination drop dries, although in the sheltered environment of the micropylar canal drying may be slow. The uptake of nonfloating pollen could otherwise be explained by the resorption of the pollination drop, or even the fluid in the micropylar canal, by ovule tissues, but resorption is difficult to prove when direct observation inside the ovule micropyle is impossible.

The observation that normal pollen always floats with its wings upward leads to consideration of pollen orientation within the micropyle. The orientation of pollen grains observed during this study were recorded and the data analysed using a Chi-square test, based on the hypothesis that the orientation of pollen grains against the nucellus was random. Tests on pollen orientation in sixteen cones showed no significant deviations from the hypothesised ratio (that one grain in six has both wings directed away from the nucellus).

Therefore, in agreement with McWilliam (1958) and Sarvas (1962), it seems that the orientation of pollen grains on the nucellus is not affected by the pollen wings. As has been suggested, pollen could be jostled by other pollen grains moving into the micropyle at the same time, or they could be moved by another withdrawal force. In many cases pollen tubes arise above the nucellus and curve towards it, so that the orientation of the grains within the micropyle does not affect the success of tube penetration of the nucellus (photographs 27E and 29A). The function of the pollen wings, then, appears to be to float the grains

through the micropylar tube, although resorption of the drop may play a part in withdrawing nonfloating pollen.

(2) The Stage of Cone Development when Pollen is Trapped and when Pollination Drops are Produced

The earliest stage at which the pollination drop is produced could be determined by sectioning cones at various stages of receptivity and recording the stage at which pollen first appears in the micropyles. However, pollen can only enter the micropyles if it is in the vicinity of the ovules when drop production occurs. Emerging cones allow the entry of only small numbers of pollen grains because the scales are still close together, not having been spread apart by the elongation of the cone axis.

Also, the time during which pollen can accumulate is shorter for prereceptive, and for this reason alone pollen available for transport into the micropyles of emerging cones is always less than in receptive cones. Thus, conclusions drawn from counts of pollen inside the micropyles of naturally pollinated cones must take this into account. The fact that low pollen numbers are found in cones at  $\frac{3}{4}E$  (figure 3:2) and increase as receptive and postreceptive stages are reached does not prove that prereceptive cones put out fewer pollination drops; they might produce them but there might not be any pollen present to mark the fact.

In artificial conditions, however, excess pollen can be applied to bagged cones for a very limited period of time so that the time of pollen arrival relative to the stage of cone development is known and there is a better chance of getting pollen into the narrower between scale channels of young cones. Pollen was applied to twenty five bagged cones and individual records of cone development were made. The cones were then left until micropylar closure had occurred and microscope counts of the number of pollen grains which reached the micropyles were made. The results (figure 3:3) indicate that the greatest number of pollen grains reaching the micropyle were in cones which were pollinated at receptivity. Two receptive

cones which received less than sixty grains in thirty ovules could have been partly obscured from pollen being directed into the bag. Eleven out of sixteen cones pollinated at earlier developmental stages received an average of more than one pollen grain per ovule.

These results are valuable because they provide evidence for tree breeders that optimal pollination is achieved artificially when pollen is applied at receptivity. When pollen is applied to cones in pre-receptive stages it can usually reach the vicinity of the ovules in amounts which are sufficient to pollinate most ovules, but the levels of pollination achieved are rather variable.

Providing the majority of ovules receive at least one pollen grain in their vicinity, the presence of pollen grains in ovule micropyles can be used to indicate that a pollination drop was formed in each at some stage prior to harvesting, assuming that pollen reaches the micropyle only by way of a pollination drop. Because of the orientation of the ovule and the micropylar canal it seems unlikely that pollen can roll or be blown directly into the micropyle in any quantity. The number of drops detected by using pollen as a marker might be slightly lower than the actual number of drops because of the restricted entry of pollen grains into prereceptive cones. But, from the pollen levels found in cones supplying data for figure 3:3, it is unlikely that many ovules would be lacking an available pollen grain and therefore the method will be used to determine the stage of development at which drop production occurs.

Figures 3:4 and 3:5 show the <sup>proportion</sup> of <sup>pollinated</sup> ovules (in a sample of thirty) from each cone at an assessed stage, which were harvested within a few days of pollen application. The variation between cones is high, perhaps because of the subjective assessment of the <sup>developmental</sup> stages, variation in the quantity of pollen able to pass into the cone channels and in the time between application and harvesting. However, figures show that a

small number of drops are produced from half emergence onward and the number is increased as receptivity is increased. This is comparable to the pattern of pollen quantity shown in Figure 3:2. Higher levels of pollen in the micropyle occurred in cones at receptivity because more grains had arrived in the cone and more drops had been produced to move them. In cones at  $\frac{1}{2}E$  all the scales bearing functional ovules are exposed.

Therefore, although a few drops are produced prior to receptivity, the evidence (figures 3:4 and 3:5) suggests that most drop production occurs during receptivity.

Because Brown (1970) suggested that bagging cones might affect the production of the pollination drop, an experiment was carried out on unbagged cones, using marked (black) pollen, applied at a recorded time and developmental stage. After a period of time, pollen was counted in a sample of thirty ovules, dissected from each cone. Comparison of the amount of yellow pollen, which must have been taken up by drops formed prior to the application of excess black pollen with the quantity of black pollen taken up, gives information on the developmental stages when drop production occurs. The data shown in figure 3:6 shows that pollen drop production had begun and was continuing in a receptive cone, but was completed, or nearly completed in a late receptive cone. Figure 3:7 shows that some naturally arriving pollen had been taken up at  $\frac{1}{2}E$  and that pollen was being taken up by more advanced cones. Yellow pollen could be taken up along with black pollen, but the amount of black pollen inside the cones was so great that the chances of yellow pollen getting into micropyles once black pollen had been applied must have been slight. Figure 3:8 shows that the proportions of yellow pollen in micropyles of cones which were left to closure was lower in receptive and late receptive cones than in the comparable cones from Figures 3:6 and 3:7. Thus, there is also considerable variation in the amount of natural pollen in receptive cones. This probably results from the variation in time that

receptive cones had been receptive prior to black pollen application. In chapter IV it will be shown that the duration of receptivity varies between two and thirteen days (figures 4:7 and 4:8) and there are no visible indications of developmental differences within the receptive stage, which lasts longer than other developmental stages. But, although precise results cannot be obtained from the technique, it provides further evidence that the main stage at which pollen drop production occurs is around the stage of receptivity and possibly through to late receptivity.

Although black pollen was applied in excess, the amount of black pollen taken into the micropyles was apparently low in cones which were left for only two days (figure 3:6). Confirmation of this would have been possible if more cones had been treated and analysed, but there was insufficient time to do this. However, cones which were left until closure (figure 3:8) had taken in much more black pollen at all stages, than those left only two days (or even up to six days), and therefore the presence of pollen in large amounts did not stimulate immediate drop production throughout the cone. The longer the interval between the application of excess pollen and the time of harvest, the greater the amount of black pollen taken up by cones at all stages is also evidence that the same ovules may produce a pollination drop on more than one occasion.

The results of all these experiments show variation between cones which have undergone the same treatment. Some sources of variation have been given. In addition, it will be shown in chapter IV that the micropylar capacity of a cone limits the amount of pollen taken in and can, itself vary. Furthermore, it is possible that the time of pollination drop production may be influenced by the environment as well as the developmental stage of the cone. However, there is evidence that most drop production occurs during the receptive period and so receptive

cones were investigated further, so that, where possible, variables could be eliminated.

Bagged cones from clone 372 were harvested at daily intervals from the time when they were all recorded at first receptivity, and the pollen in thirty micropyles was counted. On the same day of harvest an unbagged cone of similar appearance was harvested and treated similarly for comparison. Because bagged cones develop more rapidly than unbagged cones ( chapter IV, table 4:7), it was not possible to match the cones at the time of bagging. Therefore the time that naturally pollinated cones had been receptive prior to harvesting was not known. However, the amount of pollen accumulated in the cones, although not precise, probably gives a rough indication of the time that each unbagged cone had been receptive. In figure 3:9 the number of pollen grains in the micropyle of the unbagged cones is plotted against the number of pollen grains trapped by the cone and is seen to increase up to a point, where the size of the micropylar chamber is probably limiting. This suggests that naturally arriving pollen is taken up as it arrives, over a period of time. On the other hand, the pollen available to the bagged cones at the beginning of receptivity was excessive, but even so, the amount of pollen transported into their micropyles increased over a period of twelve days; it was not all taken up immediately (figure 3:10). The pattern of pollen uptake is apparently similar, whether pollen is arriving gradually (natural pollination) or is already present in excessive quantities (artificial pollination). When pollen is arriving gradually, the fact that much of it is taken up soon afterwards (possibly the night after arrival) suggests that, either the majority of ovules produce drops to ensure that transport is effected in ovules with pollen on their arms, or the presence of pollen on its arms stimulates pollination drop production by that ovule. Neither of these explanations can apply to the uptake of pollen, gradually, by bagged cones when there is excess pollen available from the beginning of receptivity.

% poll'd	days after poll'n	No. of pollen grains in micropyle.					
		0	1	2	3	4	5
5	4	38	1	1			
8	2	37	1	2			
10	2	36	2	—	—	2	
17	3	33	2	3	2		
23	4	31	3	4	2		
27	7	29	8	3			
70	11	12	25	3			
73	6	11	25	3	1		
73	9	11	16	12	1		
80	12	8	13	6	11	2	
83	8	7	31	1	1		
87	6	5	22	9	4		
95	14	2	10	14	7	7	
95	15	2	9	6	11	10	2
98	11	1	35	3	1		
98	10	1	29	8	2		
98	7	1	23	12	4		
98	12	1	14	11	9	5	
98	15	1	6	12	14	7	
98	12	1	1	4	19	15	
100	12	0	20	10	7	3	
100	13	0	7	12	15	6	
100	13	0	6	6	18	10	
100	14	0	5	8	15	10	2
100	21	0	3	15	15	5	2

Table 3:2      The number of ovules in a sample of forty, with 0, 1, 2...  
pollen grains in their micropyles. Cones were pollinated  
with excess pollen at first R. See also figure 3:10.



Table 3:2 shows the number of ovules with 0, 1....grain per micropyle in bagged cones, harvested as receptivity progressed. Early on most ovules contain one or no grains, but as they advance developmentally the number with two to four grains increases. In the bagged cones observed, pollination drops must have been produced gradually by different ovules, then later produced several times by the same ovules (a point which will be considered in section 3). Results from experiments using marked pollen show that this can happen with unbagged cones too (figures 3:6 to 3:8) so it is difficult to explain how naturally pollinated cones appear to transport pollen into their micropyles as it arrives, unless it is a result of weather conditions not operating when the bagged cones were pollinated at an earlier date. A wet period could induce drops in many of the ovules in a cone which had just reached R.

Because the pollen uptake goes on for about twelve days and the duration of receptivity in bagged cones averaged 8.2 days (chapter IV), the ovules were probably still producing drops in bagged cones at late receptivity. However, in chapter IV it will be shown that individual cones were assessed at receptivity for a period ranging from two to thirteen days, depending on weather conditions which stimulated cone closure. Cone closure did not appear to be related to the level of pollination. Because more pollination drops were observed in wet conditions, the humidity might enhance cone closure by stimulating early and simultaneous drop production in young receptive cones.

### (3) Evidence for the Continued Production of Pollination Drops by Pollinated Ovules

Doyle and O'Leary (1935) and McWilliam (1958) considered that the production of a pollination drop ceased in pollinated ovules, but Sarvas (1962) disagreed. Because of the conflict in opinion and the lack of evidence, the question will be considered here. The data in table 3:2 gives evidence for the uptake of pollen into one ovule

on more than one occasion- the longer the cones are left before being harvested, the lower the proportion of ovules with only one pollen grain. This can be seen in another way by comparing the time when all forty ovules in the cones harvested accomodate one or more pollen grains (figure 3:11) with the time when the number of pollen grains being taken into the micropyle ceases to increase - after the application of excess pollen at the beginning of receptivity. From figure 3:11 it appears that all cones had 98% to 100% of their ovules pollinated between the seventh and the twelfth day, but the number of pollen grains levelled off after the fifteenth day (figure 3:10).

(4) Examination of Ovuliferous Tissues which could be  
Associated with the Secretion of Pollination Drops

Ovules from prereceptive and receptive cones were examined with an electron microscope to see if the ultrastructure of any of their tissues showed evidence of secretory activity. E.M. studies have been made on secretory cells, such as nectary cells (Findlay and Mercer, 1971; Rosen and Thomas, 1970) and on mucus producing cells (Mollenhauer, 1967; Horner and Lester<sup>R</sup>, 1968; Schnepf, 1968 and Hyde, 1970), but no particular features have been attributed to all secretory cells. Large numbers of mitochondria were suggested by Luttge (1971) to be associated with sugar production, but he also reported large numbers of Golgi bodies (dictyosomes) in some sugar secreting cells, while in others cell wall protuberances were a feature.

A technique similar to the periodic acid-Schiff's reagent staining to show the production of polysaccharides in cells, was established by Thiery (1967), using silver instead of Schiff's reagent. Because the pollination drop has been shown to contain some sucrose, this technique could show precisely the tissues responsible for pollination drop production. The existence of this technique was not discovered until after the 1973 pollination period, so could not be used in this study.

Ovules at the beginning of cone emergence, seen with the light microscope, have a nucellus of undifferentiated cells. Cell division is rapid from this time and during emergence the ovule increases in size, the nucellus cells differentiate into a cap region, a chalazal region and the spongy cells, which surround the megaspore mother cell (photograph 17C). The cap cells are rich in starch grains, but the central outer cap cells lining the micropyle, as Ferguson (1904) noted, become elongated and clear and some appear to be collapsed and degenerating. (photographs 18 A to F).

As the cones emerge the megaspore mother cell enlarges and its end walls become pointed (photographs 17 C and 19B). Some of the spongy cells stain bright red and look misshapen, but in normal ovules of older cones there is no sign of degeneration in the spongy tissue and it seems that these cells could be passing through a particularly active stage. These 'active' spongy cells contain amyloplasts, lipid bodies, mitochondria, a few Golgi bodies and some small vacuoles (photograph 19A, top left corner and bottom right corner and photograph 19 B along one edge). The material was not well fixed and the presence of endoplasmic reticulum is not certain, but is suggested by lines of lower density. Nutrients being supplied to the megaspore mother cell must pass through the spongy cells so that their 'activity' could be associated with the nutrition of the megaspore mother cell.

E.M. sections of an ovule from a cone at the beginning of emergence show that the nucellus is made up of thin walled cells, very occasionally with darker lines crossing them. These could be plasmadesmata. (photographs are not included because they had to be limited to the most important.) The nucellus cells contain many mitochondria, a few small amyloplasts, some Golgi bodies, lipid bodies and chromatin which is often condensed. Many cells are dividing (photograph 17C).

Electron micrographs of micropylar arm cells in an ovule from a cone at  $\frac{3}{4}$ E showed vacuoles of varying size, containing dense material

not seen in the rest of the ovule. These can be seen with a light microscope as dark patches (photographs 18A to F, in the upper corners), but are not apparent in material fixed in F.A.A. so the contents of the vacuoles are possibly soluble in alcohol.

Cells in the chalazal region of the nucellus of ovules in receptive cones contained many lipid bodies, mitochondria and some amyloplasts, while cells of the nucellus cap appear to be richer in amyloplasts, although lipid bodies, mitochondria and small vacuoles are also present.

In some cells in the edge of the nucellus cap in cones which had been receptive for several days, concentric rings of lamellar endoplasmic <sup>reticulum</sup> surround cell organelles (lipid bodies, amyloplasts or mitochondria) and enclose pockets of cytoplasm (photographs 20A and B, 21A and 22B). Large numbers of ribosomes and numerous Golgi bodies are present. Other cells containing endoplasmic reticulum and many ribosomes, also contain enlarging vacuoles (photograph 22B). Still other cells, the clear cells seen with the light microscope, contain vacuoles which fill most of their volume, confining the cytoplasm and organelles to the periphery of the cell (photographs 23 A and B). Degenerating cell contents are seen in the vacuoles. Among the vacuolate cells, collapsed cells could be seen (photographs 21B, 22B and 23A and B). Amyloplasts, degenerating chromatin and areas of lamellar endoplasmic reticulum could be identified in them (photograph 21B).

When receptivity is reached, irregular spaces form in the vacuolate cells, between the cytoplasm and the cell wall (photographs 21A and B and 22A and B). The development of the nucellus cap cells from undifferentiated parenchymatous cells to cells containing many ribosomes, and organelles surrounded by lamellar endoplasmic reticulum and eventually to vacuolate and collapsed cells is suggestive of a specialized function which is completed during receptivity. Their position, border-

ing the micropyle suggests their association with pollination drop production.

Light microscope photographs 18A to F, from the same ovule from which electron microscope studies were made, show sections one micrometer in thickness. These were cut at approximately ten micrometer intervals, from the edge of the nucellus towards the centre. On the outer edge the cells are full of cytoplasm with only small vacuoles in some. Towards the centre, cells with very large vacuoles appear and increase in number, but in the very centre the cells had collapsed. Thus, it is possible that the cells in the centre began to secrete the pollination drop first and the outer nucellus cap cells contribute to pollination drop production later. This sequential pattern of cell development could account for the continued production of pollination drops by one ovule over a period of time.

#### (5) Cone Colour of *Pinus radiata* over the Pollination Period

Cone colour in *P. radiata*, from emergence to cone closure, has been described in table 3:1. In most clones it ranges from pink at emergence to purple at closure (photographs 14 A to D and 15A and B). However, at each stage, different clones may show variations in the depth of colour. Cones of one clone are lacking in the red pigment and are green (G.B.Sweet, pers. comm., 1970).

About four to six weeks after cone closure the cones reflex from their upright position and three months after cone closure they become green.

## IV. DISCUSSION

#### (1) Ovule Structure and Pollination Drop Production in *Pinus*

Pollination in *Pinus* is a complex physiological process, requiring precise timing of drop synthesis and release with structural receptivity and pollen shed. Pollination drops have been reported in other

families besides the Pinaceae (Tison, 1911; Doyle, 1945). They also occur, within the family, in other genera than Pinus and although it was thought that Larix, Cedrus, Tsuga, Abies and Pseudotsuga did not produce pollination drops (Doyle, 1945), Barner and Christiansen (1960) showed that they do occur in Larix, but it is not until five to seven weeks after pollen is trapped in the cones. This suggests that the second stage of pollination, transport into the micropyle is delayed. The pollination mechanism was, therefore, probably established in conifers before the evolution of the genera of Pinaceae, and it is therefore likely that the structure of the ovule and the mechanism of pollination is similar between species of Pinus. It has been shown here that ovule structure, tissue appearance and the occurrence of the pollination drop are alike in all species described. In these respects, then, P.radiata is like the species described and illustrated by Ferguson (1904), Sethi (1928), McWilliam (1958), Sarvas (1962), Burley et. al. (1967) and Register and West (1970). Therefore, the details of pollination drop production and pollination, except for time intervals, probably apply to other species of Pinus also.

## (2) Pollen arrival in the Cone and the Pollination Drop

This study has shown that pollen can be trapped in small amounts by the cone as it is emerging, and in greater quantities once the cone scales are wide apart. Prior to receptivity some ovules are able to exude pollination drops, but in bagged cones most ovules produce drops after receptivity is reached. Ovules continue to put out pollination drops during receptivity and even when the late receptive stage is reached, if the micropyles are not already full. It is not known if drop production continues when the micropyle is full.

It is possible that the volume of the pollination drop is enhanced by humid conditions and that it does not always well out beyond the micropyle rim. In bagged cones pollen was often taken up one grain at a time

in the first few days of receptivity, even though there was plenty of pollen on the integument arms. Pollen drops were rare in unbagged cones observed at intervals one night, but small ones may not have been noticed.

### (3) The Mechanism of Pollen Transport

Pollen grains are able to enter the micropyle even when they are treated in such a way that they are unable to float in a drop of water. They must in that instance be carried by the movement of the fluid, resulting from its active absorption by the ovule tissue, or by its evaporation and the recession of its surface. Neither observations nor experiments carried out in this study showed conclusively which process occurs, but, because the internal surfaces of the cone are very moist and the micropylar canal has only a small opening, it is hard to see how the drying of the drop could supply a force big enough to transport pollen up into the micropyle. On the other hand, if cells producing the pollination drop collapse during or after its release, it would not be expected that they would be capable of resorption of the drop, though other ovule cells may be.

However, under natural conditions, the pollen probably floats through the pollination drop, up through the micropylar canal and into the micropyle, where it lodges against the nucellus tip and germinates. The process of germination will be described in chapter IV.

### (4) Synthesis of the Pollination Drop

Although there is evidence that the pollination drop is secreted, the physiological processes associated with it cannot be interpreted from the study of fixed material. The sequence of changes in the nucellus tip cells and the fact that a large number are collapsed by the time the cone closes, suggests that their function is associated with pollination. These cells breakdown well before germination commences so their collapse is not due to the penetration and absorption by the pollen tube. The development of additional Golgi bodies, endoplasmic reticulum and ribosomes might be associated with production of pollination drop components.

## CHAPTER IV

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## CHAPTER IV

## POLLINATION IN THE STAND

## I. INTRODUCTION

## (1) Purpose of the Study

Pollination can be regarded as being successful when all the ovules of a cone contain micropyles which are filled to capacity with pollen grains. If successful pollination is to occur two conditions are necessary: firstly, there must be sufficient pollen in the air to fill all the ovule micropyles, and, secondly, the cones themselves must be able to trap the pollen and move it into the micropyles.

In the literature the coincidence of pollen shed and cone receptivity is not generally considered. Data on pollen shed in pines is plentiful (Fielding, 1957; Ebell and Schmidt, 1964; Koski, 1970), but data on cone receptivity is scant (Zobel and Goddard, 1954; Sarvas, 1962).

Sarvas (1962) showed that P.sylvestris ovules aborted because pollen failed to reach them, and he carried out detailed studies on pollen shed, which he related to pollination levels in ovules from the same sites. Bramlett (1973) found that a large percentage of the ovules of P.echinata from the tip or lower border of the ovule bearing zone aborted because they were in an unfavourable position to receive pollen. Nekrasova (1970) showed that the last P.siberica cones to become receptive received insufficient pollen; often as many as 62% of the ovules were unpollinated. The relative success of pollination, therefore, affects the seed production of pines and there may be several factors affecting the success of pollination.

The relationship of cone receptivity to pollen shed in P.radiata and the success of its pollination in different years, clones and sites are of importance to the tree breeder and seed orchard manager and this constitutes the reason for carrying out the study reported in this

chapter. The remainder of the introduction will examine the literature on some of these factors in P. radiata and other species.

## (2) Variation in the Commencement and Duration of Pollen Shed

The commencement date of pollen shed and its duration, relative to the time of receptivity of cones in the same stand, could affect the overall success of pollination in any year.

Commencement of pollen shed in any Pinus species varies with the year, altitude and site. The variation has been related to climatic factors, particularly temperature and evaporation (Millet, 1944; Buell, 1947; Florence, 1958; Sarvas, 1962; Ebell and Schmidt, 1964). These factors also affect the duration of pollen shed and the pattern of dispersal throughout the period. Millet showed that the duration in P. radiata, at one site, varied from four to ten weeks in different years and from two to three weeks at another site. At another locality, P. radiata pollen was trapped from the atmosphere over a six week period and individual trees were seen to release pollen over nineteen days (Fielding, 1957). Duration of shedding for some other species at other localities range from a few days (P. siberica, Nekrasova, 1970) to two weeks (P. contorta, P. monticola and P. elliottii, Ebell and Schmidt, 1964; Florence, 1958), and four weeks (P. gerardiana, Konar, 1962).

## (3) Pollen Quantity and the Pattern of Pollen Shed

The quantity of pollen at one site can vary from year to year and can also vary from site to site (Koski, 1970). More pollen is produced on good quality sites than on low nutrient sites (Florence, 1958; Sarvas, 1962). But, within a stand in one season, pollen production can vary greatly between trees (Sarvas, 1962; Bramlett, 1973).

Within a pollination period the quantity of pollen being shed varies. Pollen shed usually begins slowly, reaches a peak and tapers off, but considerable variations, due to weather effects, are superimposed on this pattern (Fielding, 1957; Sarvas, 1962; Koski, 1970).

Florence (1958) and Bramlett (1973) showed that the pattern of pollen shed varied from year to year, with even dispersal in some years and marked peaks in others. In some years the days of peak pollen shed were not consecutive. Sarvas (1962) found no marked peak during a pollination period when the total quantity of pollen shed was low.

A diurnal pattern of pollen shed with maximum amounts recorded at midday or early afternoon and an almost complete cessation at night was described by Fielding (1957) and Sarvas (1962). The latter pointed out that this pattern was related to daily changes in humidity. Since most cones are receptive for more than a day, the pattern of diurnal pollen release would not be expected to affect the success of pollination.

#### (4) Dissemination of Pollen

When seed orchards were being established, research on the dispersal distances of pollen was carried out to determine the best size for a seed orchard and the degree of isolation it needed. The dispersal of pollen was estimated from the amount of pollen trapped at different distances from a pollen source (Buell, 1947; Sarvas, 1962; Boyer, 1966). Other estimates of pollen dispersal were made by detecting radioactive pollen released from one point (Colwell, 1951; Koski, 1970). Early results suggested that most pollen fell within a short distance. According to Wright (1953) this was because pollen wings contract in dry air, but it has been pointed out (chapter III) that wings probably do not affect pollen flight. Furthermore, large pollen clouds have been detected at considerable distances from the pollen source (Lanner, 1966<sup>b</sup>). Lanner considered that previous experimental work had been carried out under conditions of a horizontal wind force. He described the alternative movement of air masses, which occurs in forests, whereby pockets of heated, moistened air may be carried up to considerable heights through the main air masses. If pollen was trapped in one of these thermal shells it would not be released until the shell was dissipated.

Measurements showing long distance pollen dispersal and increased pollen concentrations at greater heights have been described by Koski (1970). But, he also observed that it is the pollen from the immediate vicinity of a tree which effects most of its pollination. Boyer (1966) found that rain reduced pollen shed.

Probably the pollen concentration falls off sharply within a short distance of its source, but the pollen carried upward maintains a concentration for hundreds of miles.

#### (5) Commencement and Duration of General Cone Receptivity

Cone receptivity is a developmental stage which lasts over a period of time, and it is necessary to qualify its nature. Individual cones are receptive for a short period. In the seed orchard, however, between clones and within clones, cones reach receptivity at different times. There is, therefore, a much longer period of time during which at least some cones in the seed orchard are able to trap pollen. This will be referred to as the period of general receptivity of the orchard. The period during which a clone bears one or more receptive cones is the period of clonal receptivity and the receptive period of single cones will be called individual cone receptivity.

Commencement of general cone receptivity, like that of pollen shed, varies with year, site and altitude. At one site the difference between years was up to eight days for P.elliottii and at another site up to four weeks for P.echinata (Zobel and Goddard, 1954). However, these are extremes and, at other sites, P.echinata showed less variation in the onset of receptivity. The difference between commencement dates for other species lay between the two extremes given for P.elliottii and P.echinata (Zobel and Goddard, 1954; Nekrasova, 1970).

Similarly, the duration of receptivity varied with year and site, ranging from nine days to four or five weeks in each species (Zobel and Goddard, 1954). The duration of receptivity for P.siberica was eight

to twelve days (Nekrasova, 1970).

Warm weather conditions accelerate the process of cone development so that the duration of general receptivity in warm weather is short (Ehrenberg and Simak, 1956).

#### (6) Individual Cone Receptivity and its Variation Between Trees

Information on the receptive periods for individual cones is sparse and general. In P. palustris, P. elliottii, P. echinata, P. taeda, P. sylvestris and P. siberica the individual cones remain open for a few days only (Zobel and Goddard, 1954; Sarvas, 1962; Nekrasova, 1970). However, receptive cones could be found on a single P. echinata or P. taeda tree often for a two week period and, in some cases, for three or four weeks (Zobel and Goddard, 1954). Fielding (1960) reported that receptive cones on one P. radiata tree were present for two months or more and pointed out that the polycyclic character contributed to this. He also considered that cones receiving most insolation became receptive first. Sarvas (1962) noted the same effect, but in P. sylvestris the difference in timing between cones on one tree was only one day. He also found that the difference in timing between individual trees was not more than a few days. However, there were considerable between tree differences in P. radiata (Fielding, 1960).

#### (7) The Relationship of Cone Receptivity and Pollen Shed

In general, pollen dispersal and maximum cone receptivity are considered to be synchronous (Zobel and Goddard, 1954; Bramlett, 1973). The commencement of both cone receptivity and pollen shed, each year, was affected by temperature in the preceding few months (Zobel and Goddard, 1954; Sarvas, 1962). However, Fielding (1960), in Australia, noted that the first female cones in P. radiata emerged before pollen shed commenced and Nekrasova (1970) made the point that the latest cones of P. siberica are poorly pollinated. Furthermore, Sarvas (1962) considered that weather conditions affected the relationship of pollen shed to cone receptivity

and that, when conditions produced rapid pollen shed, they commenced at the same time. But, changeable, humid conditions could delay pollen shed to a greater extent than cone receptivity. According to Zobel and Goddard.(1954), extremes of weather did affect receptivity of P.taeda so that in cool conditions female cones were slow in reaching receptivity, while the male cones continued to shed pollen so that there was little left when the cones were receptive. The apparent contradiction in the findings of these authors might be explained if the relative humidity was low during the cool period described by Zobel and Goddard.

(8) The Amount of Pollen reaching the Ovules' Integument Arms and arriving in their Micropyles

A few brief statements as to the number of pollen grains usually found in ovule micropyles have been given in the literature. These are best summarized in a table.

Species	Author	Usual no.	Maximum no.
<u>P.virginiana</u>	Thomas (1951)	2 to 3	5
<u>P.sylvestris</u>	Sarvas (1962)	1 to 2	5
<u>P.sylvestris</u>	Brown (1970)	2 to 3	6
<u>P.siberica</u>	Nekrasova (1970)	1 to 2	3

Table 4:1      Reported number of pollen grains contained in micropyles of Pinus species.

Sarvas (1962) showed that in good years the amount of pollen arriving in the vicinity of the average ovule of P.sylvestris was 7.9 grains, with a maximum of about 20 grains. Conversely, in a bad year there was an average of only 1.9 grains per ovule.

Although Sarvas stated that the unit of pollination was the female cone, he took his pollen counts from samples of ovules from many cones. His data give an overall measure of pollination in the

stand, but do not show differences between pollination units (cones). Therefore, the effects of the timing of individual cone receptivity on the success of pollination remain unknown and the relative success of pollination in different clones cannot be determined.

#### (9) Artificial Pollination

Techniques of artificial pollination have been described (Cummings and Richter, 1948), and results in terms of successful seed production have been given (Ehrenberg and Simak, 1956; Brown, 1970). Ehrenberg and Simak showed that the earlier the bagged cones were pollinated, the smaller the cones and the less the seed set, but pollination at peak receptivity resulted in well developed cones and seed set comparable to that obtained after open pollination. In the previous chapter P. radiata cones were shown to be well pollinated when the pollen was applied at receptivity. Pollen applied prior to this was able to get into the cones, but the number of pollen grains per ovule was reduced. Pattinson et al (1967) found that controlled pollination could be carried out from the time the cones emerged from their bud cataphylls to the time the ovuliferous scales began to close, in P. kesiya. However, in his work with P. sylvestris, Brown found that a high proportion of ovules in artificially pollinated cones had not received pollen. He considered that the force of pollen application, the lack of circulation of pollen in the bag and condensation in the bag could affect the amount of pollen arriving inside the cone. He also suggested that bagging might upset pollination drop production, because air temperatures in the bag were more extreme than external air temperatures. But, Brown also suggested that the most important factor affecting the success of artificial pollination, was the timing of pollen application.

#### (10) Conclusions

A consideration of this literature shows that there is very little information on the variability in receptivity of female cones in the



stand, or on the factors which ensure or inhibit the successful pollination of female cones. Such information is necessary for the management of P. radiata seed orchards. For this reason the relationship of pollen shed to commencement and duration of general cone receptivity, clonal receptivity and individual cone receptivity will be described. In addition, factors affecting the duration of individual cone receptivity, the amount of pollen reaching the integument arms, and the amount of pollen accommodated inside the micropyles will be considered. Finally, the success of artificial pollination will be examined.

## II. MATERIALS AND METHODS

### (1) The Material

The material used for the measurement of pollen levels in cones came from the site at Kaingaroa, described in chapter II and from the site at Whakarewarewa, described in chapter III. Daily observations of receptive cones were made at Whaka.

Pollen counts were made from both sectioned material and fresh dissections, which were described in the previous chapter. In 1972 daily observations were made on forty cones from each of six clones in the clonal archive at Whaka. This meant that the clones chosen had to include trees which were strong enough to withstand being climbed. Clones 274, 372, 89 and 246 had been used as experimental trees in 1971, so there was already data available for them. Clones 19 and 55 were included because they were the clones harvested at Kaingaroa and this made some site comparisons possible.

Results from clone 246 have not been discussed in this thesis because estimation of its stages of receptivity was complicated. The small, round cones from clone 246 did not emerge fully from the bud cataphylls at the time they were receptive. This meant that they were recorded at  $\frac{3}{4}$ E to EFE, when, in fact, they were receptive. This was

not realized at the beginning of the study so the duration times calculated during the analysis were not comparable to those of the other clones observed.

Clones 55, 274, 372 and 89 bore either one or two cycles of cones on their first order branches. Clones 19 and 246 were less vigorous trees and bore only single cycle cones, except on their terminal shoots where there were two cycles of cones. Clones 55, 274 and 89 bore three cycles of cones on the leading shoot and clone 372 bore two. The cones at the tip of the leading shoot could not be reached by climbing and so were never observed or harvested. Third cycle cones probably make up a relatively small proportion of the total cone crop and seem to occur on the early receptive clones. (This requires verification from detailed observation of a large number of clones.) If the suggestion is supported, then third cycle cones on early receptive clones are probably receptive at the same time as second cycle cones on late receptive clones, and, therefore, are probably pollinated with comparable success.

## (2) Observation in the Field

First and second cycle cone clusters on the observation trees were labelled and numbered. Forty first cycle cones were inspected for each clone and twenty second cycle cones (because they were generally fewer) were inspected for clones 55, 89 and 274. All cones from clone 55 were sampled from one tree and in clone 89, 274 and 372 they were sampled mainly from one tree, but a few cones were supplied from a second tree. Clones 19 and 246 were less vigorous and bore fewer cones, so four trees were sampled. In 1973 the same trees (except clone 246) were visited for the later part of the pollination period only, so records were not taken in such detail.

At each visit the degree of receptivity of each cone in each cluster was assessed and recorded so that the overall receptivity of the

tree (s) could be shown along with the developmental changes in the individual cones. A proportion of the cones with a recorded history were harvested. These were either dissected ( and counts made on 40 ovules) or were preserved in F.A.A. for microscopic examination.

In 1973 late receptive cones from clones 19 and 55 were sampled and thirty ovules dissected. The number of pollen grains in the cones per forty ovules and in forty micropyles was calculated from these data so comparisons could be made with 1972 results.

### (3) Recording the Time and Quantity of Pollen Shed

It is necessary to study pollen shed because it affects the success of pollination, but it is only one aspect of this study of the whole development of the female cone. Fielding (1957), Sarvas (1962), Koski (1970) and others measured pollen shed by setting up pollen traps and later counting the pollen. It was, however, impractical for one person to do this when so much other data had to be obtained in the same few weeks.

To give some idea of the commencement and duration of pollen shed, forty four clones were inspected at intervals of several days. This was done by walking along the rows and assessing whether any pollen was being shed for each clone. This does not give a precise measure of the quantity of pollen in the air because of variation between and within clones in the quantity of pollen released. Grafts with only a few pollen bearing twigs often have very few male cones on those twigs and tend to shed their pollen over a short period. Grafts with abundant pollen may continue to shed it for at least twelve days. In these cases pollen shed begins slowly in the uppermost pollen bearing branches and is followed by pollen shed in the lower branches. Because of this, each tree contributes variable amounts of pollen over a period of time. Therefore, at the beginning of pollen shed, although 5% of the clones might be shedding pollen, some of these might have little to contribute and

most might be shedding small amounts from the upper branches only. This means that the amount of pollen being shed is much lower than 5% of the potential amount. Nevertheless, the date when some pollen was available at the beginning and end of pollination, and the time when most clones were shedding pollen gives some information on the progress of pollen shed.

Sarvas (1962) did show a linear relationship between the amount of pollen trapped in globe meters and the amount of pollen on ovules from the same stand. In the present study counts were made of the amount of pollen in the vicinity of forty ovule micropylar arms, dissected from cones of known clones at recorded times. The forty ovules were sampled in the same way for each cone. The ovule bearing zone was visually divided into five zones, but fewer ovules were sampled from the upper two zones where the cone tapered. The zones with their sample sizes given in brackets were; tip (6), lower tip (7), upper middle (8), middle (10) and low (9). Grains in the vicinity of the forty ovules included all grains on the micropylar arms and rim, in the micropylar canal and those accommodated in the micropyle itself. This was considered to be a representative proportion of the pollen grains arriving in the cone and so the cones could be treated as living pollen meters. The advantage of this was that a realistic measure of the amount of pollen entering a cone and available for ovules could be shown.

Disadvantages arise from some uncontrollable variables, which probably affect the amount of pollen entering each cone. The position of the cone on a tree, its height, aspect and coverage by vegetative material are some. Since only one or two cones could be dissected each day, these effects could not be investigated. Cones may remain receptive for a few days to a week or more and the pollen accumulates in them over this time. Thus, variation occurs in the relative time a receptive

cone has been left prior to harvesting. Records of individual cone development gave a measure of this variable. It is known that weather conditions affect pollen flight, producing a variable pattern of pollen arrival over a period of time. General weather conditions have therefore been noted for each date of harvest. Furthermore, clonal characters, such as cone size, shape and perhaps developmental times, could influence the amount of airborne pollen which passes into them.

As long as these variables are kept in mind, the data from many cones, harvested over a period of time, is of value in interpreting the time and quantity of pollen shed as it affects the success of pollination of female cones.

#### (4) Estimating the Pollen Level in Heavily Pollinated Cones

Most data on pollen trapped in the vicinity of ovules was obtained by counting the pollen grains seen in sections of whole young cones. But, at high levels of pollination pollen grains occur in large clusters around the micropylar arms. Grains outside the ovule cannot be counted individually, but it is known that one pollen grain usually appears in three sections cut at 10 micrometers. Thus, the number of pollen grains in clusters outside each ovule were counted on every third section and the total for each ovule added to the number accurately counted inside the micropyle. The highest estimates were in the range of twenty to twenty five grains per ovule in one cone. Counts of pollen on the micropylar arms of dissected ovules confirmed that in well pollinated cones twenty or more pollen grains could be seen on the micropylar arms and around the micropylar rims of the ovules. In 1972 pollen counts were obtained by fresh dissection and also from microscope sections. When the pollen level in these cones was compared by analysis of variance, no significant difference was detected in the counts obtained by the two methods.

### III. OBSERVATIONS AND RESULTS

#### (1) Organization of the Material

Because quantitative data on pollen shed has been derived from pollen counts in cone samples, the influence of aspects of individual cone receptivity on the pollen trapped must be considered. But, the effects of cone receptivity on the quantity of pollen trapped have been determined from counts of pollen which arrived in recorded conditions. For clarity these interrelated aspects of pollination have to be considered separately.

Therefore, the general aspects of pollination in the observation stand, (duration, patterns and synchrony of general receptivity and pollen shed in 1972) will be described first. Then quantitative measurements of pollination success will be given for two clones, several years and two sites. This provides a range of pollination levels with which pollen counts from individual cones, pollinated in recorded conditions can be compared. Some interpretations of the factors affecting pollination and comparisons of the success of artificial pollination with natural pollination will be made finally.

#### (2) Commencement, Duration and Pattern of Pollen Shed and General Cone Receptivity at Whakarewarewa in 1972

Data from 1972 (obtained by counting the number of clones in a sample of fortyfour, which were shedding pollen) shows that pollen shed began before the 20th August, <sup>e</sup>reached a peak around the 6th September and was still being shed in small quantities by 35% of the clones on the 18th September, when records were terminated (Figure 4:1). If pollen shed was completed by the end of September, its duration would have been about six weeks.

Receptive cones from clones 274 and 372 were used as pollen meters to supplement this data. They were harvested on a series of days and the amount of pollen in the vicinity of forty ovules was counted. These two

clones were selected from among the observed clones because in both 1971 and 1972 clone 274 was among the earliest clones to produce receptive cones and clone 372 cones generally became receptive later. Together these clones produced receptive cones in two cycles throughout the observation period.

Results show that cones receptive on the 17th August had received very little pollen and the amount of pollen trapped in other cones did not increase greatly up to the 30th August. This period included five wet or humid days which probably limited the period of pollen flight. However, most receptive cones harvested between the 4th and 16th September had received greatly increased supplies of pollen (figure 4:2).

From the data in figure 4:2 it can be calculated that the best pollinated cone from clone 274 prior to the 30th August was harvested on the 28th August and received  $1/13$ th the amount of pollen trapped by a receptive cone harvested eleven days later. It is not possible to compare the increase in pollen trapped by clone 372 cones during the first ten days of pollen shed because no cones were receptive at this time. But, if it is assumed that there is no major difference in the ability of the two clones to trap pollen, it can be shown that the highest level of pollination in clone 274, prior to the 30th August (cone harvested on 28th August) was  $1/13$  that trapped by a cone of clone 372 on the 4th September and  $1/15$ th that trapped by another on the 15th September. Therefore, although about  $\frac{1}{3}$  of the clones were shedding pollen by 30th August, the density of pollen in the air at the peak of pollen shed (some time between the 4th and 16th of September) must have been at least thirteen times as great as it was in the first fortnight of pollination, although only three times as many clones were contributing.

The pattern of pollen shed in 1972 shows a slow beginning prior to the 17th August, with contributions from an increasing number of clones. From the 4th September, at the commencement of a period of warm,

breezy weather, the amount of pollen in the air increased and the rate of increase in the number of clones contributing was at its greatest. But, although the number of clonal contributors dropped off between the 6th and 11th September, the pollen density in the air remained high. The density of pollen was reduced after the 16th September, but pollen was still present in low amounts in receptive cones on the 21st September.

Recognition of stages of receptive development of individual cones was based on the description given in table 3:1. Figures 4:3 to 4:5 show the clonal proportions of cones classed in each developmental category, at weekly intervals.

Within clones the variation in cone receptivity can be considerable. For example, in clone 55 some first cycle cones were receptive when others were just emerging (Figure 4:3, 23rd August, 1972), and when the majority of second cycle cones were still enclosed in their bud cataphylls. Usually the most advanced cones on one tree occur on the most vigorous first order branches, while the latest first cycle cones occur on short, younger first order branches at the top of the tree. In this case the higher insolation at the tree top has not accelerated cone development, as described by Sarvas (1962) and Fielding (1960). Perhaps the nutrient status of young first order branches near the leading meristem is lower than that of older first order branches and counteracts the effect of greater insolation during the first year that such branches bear cones.

The clones appear to differ also in the relative times that their first and second cycle cones develop. In clones 89 and 274 the most advanced second cycle cones were recorded at the same stages as the latest first cycle cones. In clone 55 there were only a few second cycle cones which were at the same stage as the youngest first cycle cone (figure 4:4, 30th August).



The overall clonal receptivity (i.e. the date when each clone showed its peak percentage of cones at receptivity) was most advanced in clone 274, then in 55, 89, 372 and 19 in that order.

The gradation of time of reaching clonal receptivity in these observed clones is assumed to approximate the gradation in time of reaching clonal receptivity shown by the majority of clones at Whaka site. However, the proportion of the total clones represented by each observed clone is unknown. Furthermore, the extremely early and late clones are not represented here, although knowledge of the relative success of their pollination is of importance to seed orchard management. A separate study of the extreme clones, based on similar techniques would be useful, but could not be carried out here.

From the observations of two hundred and sixty cones from the five clones, the percentage of cones which were receptive on each day can be calculated. This is shown in figure 4:1, so it can be compared with the pattern of pollen shed by clones. Receptivity at the site began before the 15th August. As more cones became receptive in the following period (two weeks in the observation clones), the proportion of receptive clones increased steadily until the earliest receptive cones passed into the late receptive stage. This balanced the number of additional cones which became receptive and so a plateau appears in the graph. The decline in the proportion of receptive cones on the 11th September occurred because the number of cones reaching receptivity was lower than the number passing into the late receptive stage. The last receptive cone seen on the observed trees was on the 21st September. Thus, the duration of general cone receptivity was greater than five weeks and two days.

These observations suggest that a small proportion of cones in a stand or seed orchard become receptive when the pollen density in the air is low. Because information on the termination of pollen shed is

incomplete, it is not possible to say whether cones which become receptive late in the pollination period are also subjected to limited pollen supplies, but this is certainly possible.

The pattern of cone receptivity in the observed clones has been described as rising to a plateau, but it shows indications of bimodality. The first peak is around the 29th August when pollen was shown to be trapped in lower quantities. The cones contributing to this peak were first cycle cones. However, the cones becoming receptive during the period between peaks were from both cycles. Late first cycle cones and second cycle cones contributed to the second peak on the 11th September.

The cones supplying this data were taken from five clones, which reach peak receptivity at different times and which commence second cycle cone receptivity at different times. Since the second cycle cones made up only one eighth of the total cones observed, it is difficult to attribute this second peak to the increased number of second cycle cones, alone reaching receptivity. Alternative factors influencing cone receptivity will be discussed in section 5, in connection with factors affecting the duration of individual cone receptivity.

It was shown that pollen meters (receptive cones) received plentiful supplies of pollen from the 4th September to about the 16th September. It has also been shown that after the 16th September only 10% of the observed clones (second cycle cones and first cycle cones of clone 372) were still receptive and that in the following four days most of these passed into the late receptive stage. Even though some cones on the site would be receptive later than this, their proportion relative to the total number of cones at the site would be very low. Therefore, in 1972, at Whaka, 30% of the cones were receptive prior to the period of peak pollen shed, but the proportion of receptive cones dwindled about the same time as pollen shed diminished. In subsequent sections the success of pollination in cones receptive at different times will be discussed.

(3) Differences in the Relationships of Clonal Receptivity  
Between Years and in the Timing of General Receptivity

Records of the order of development in a group of clones from Kaingaroa over three years (A.Firth, Forest Research Institute, pers. comm.) showed that the order within the group can change from year to year, but the approximate position within the group is maintained (table 4:2).

Year	1966	1967	1968
Clone	121	121	121
	7	7	7
	96	96	99
	19	55	96
	55	19	82
	99	99	55
	82	82	19

Table 4:2 The order in which clonal receptivity was reached by a sample of clones from Kaingaroa in three years.

These data were not accompanied by precise measurements of the time lags between the clones sampled, or of the extent of variation in timing within clones between years. However, the differences in timing shown by the clones studied at Whaka in 1972 and 1973 was documented.

Figure 4:6 shows the pattern of clonal receptivity for clones 19, 55, 89, 274 and 372, recorded on the 24th August 1973. The sample size was variable because the cones on the same trees observed in 1972 were more difficult to reach in 1973. Clone 372 bore fewer cones in 1973. However, the patterns of clonal receptivity in 1972 which were most similar to the clonal patterns for the 24th August 1973 are shown for each clone. A comparison of the difference between 1972 and 1973 dates shows that development in clone 274 was nineteen days ahead in 1973, while

clone 55 was ten days ahead of 1972 development in 1973. The smallest difference between years (six days) was shown by clone 372. Plotting the clonal data altogether on one graph gives an estimate of general receptivity at the site. The comparable pattern for first cycle cones appeared about thirteen days earlier in the year in 1973 than in 1972, but the difference in developmental timing of second cycle cones in the two years was only six days. (The second cycle comparison has not been shown graphically.)

These results suggest that clones and cone cycles respond to environmental conditions of each year to varying degrees. In the year when general receptivity was earlier (1973) the clone with the most advanced pattern of receptive development (274) was advanced to a greater extent, relative to other clones. In the same year the latest clone (372) showed the smallest between year difference. The timing of receptivity of second cycle cones also was affected to a lesser degree than the timing of first cycle cones. Taken together, these observations suggest that later emerging cones are less sensitive to the environmental factors which influence cone emergence and receptive development. As a result, the duration of general receptivity is possibly increased by at least thirteen days when the pollination year is earlier. (The earliest clone was nineteen days ahead developmentally and the latest was six days ahead. Assuming that the duration of receptivity of the latest clone is much the same in the two years, then the overall duration of receptivity in the early year will be increased by thirteen days.) There is no detailed data on pollen shed to show whether the duration of pollen shed is similarly affected.

Mean monthly temperatures for Whaka ( Appendix 2) show that the summer of 1971-1972 was relatively cool and autumn temperatures were lower than usual. There appears to be a relationship between the mean monthly temperatures prior to pollination and the time of general cone

receptivity. Similar observations were made by Zobel and Goddard (1954). Sarvas (1967) used a concept of degree days (i.e. the number of days which exceeded a temperature of  $5^{\circ}\text{C}$  in Finland, prior to cone receptivity and pollen shed) and showed that, on one tree, female cone opening occurred within a certain range of degree days. In a population, cone opening and pollen shed occurred over a much wider range of degree days, but pollen shed, although it began later than cone opening, overlapped it to a considerable degree. The concept of degree days could be used to determine the relationship of temperature to the onset of pollination in different years so that more accurate prediction of the onset of pollination would be possible.

#### (4) The Quantity of Pollen Trapped

A quantitative measure of how much pollen can be trapped in cones under different conditions can be related to the amount of pollen taken into the micropyles of each cone. The data on pollen quantities can be compared between years and sites.

Whole cones sectioned in Kaingaroa samples from 1970 showed very high levels of pollination. In six cones of clone 55 (1970) the mean number of pollen grains in the vicinity of each ovule ranged from eighteen to twenty four, but in similar cones from 1968 the mean number of pollen grains per ovule per cone ranged from eight to nineteen. Clone 19 cones generally trapped fewer pollen grains than cones of clone 55, but also provided some high pollen counts in these years. At Whaka the highest pollen counts were made from clone 274 in 1972 ( an average of eighteen to twenty grains per ovule per cone). However, these occurred in only two cones out of ten. It is more usual for cones to trap an average of five to nine pollen grains per ovule, but it has been shown that this<sup>is</sup> enough to ensure that most of the micropyles take in two or three pollen grains and so provide a 'selection' of genotypes for the future embryo. The lowest concentration of pollen found in a develop-

ing cone was from clone 274 at Whaka (1971). Only one grain was found for every two ovules.

Statistical analysis of the quantity of pollen trapped in cone samples was carried out. From the pollination data plotted in figures 4:2, 4:7, 4:8, 4:10 and 4:11 the mean values given in table 4:3 were calculated, and comparisons were made between sites, years and clones, using analysis of variance and F tests. The details of the analyses are shown in Appendix 3.

Year	Site	Clone		
		55	19	274
1968	Kai.	488	286	-
1970	Kai.	841	530	-
1971	Kai.	231	322	-
1971	Whaka.	172	144	112
1972	Whaka.	349	-	350
1973	Whaka.	272	189	-

Table 4:3      The number of pollen grains trapped in the vicinity of forty ovules per cone (mean of five cones).

Two factor analysis showed that in 1971 there was a significant (1% level) <sup>site</sup> difference in the amount of pollen trapped by clones 19 and 55, but there was no difference shown between <sup>clones at</sup> the two sites (Kaingaroa and Whaka.). A clone-site interaction was detected (5% level).

Two factor analysis detected significant (1% level) year differences in pollen arriving in clones 274 and 55 at Whaka in 1971 and 1972, but no clone differences or clone-year interaction were shown.

Highly significant between year (0.1% level) and between clone (0.1% level) differences were shown at Kaingaroa for clones 19 and 55 in 1968, 1970 and 1971. Also, there was a significant clone-year interaction (0.1% level) which was generated by an anomalous pollination

level in one of the clones in 1971.

The cone size and degree of opening of the scales could hinder the entrance of large quantities of pollen to clone 19 (which usually traps less pollen than clone 55) or the duration of its receptive stage might be shorter. Even proximity to a high pollen contributor could result in higher levels of pollen being trapped in some trees.

(5) Variation in the Duration of Individual Cone Receptivity and Factors Affecting it.

Information on the duration of general receptivity has been related to the pattern of pollen shed, since synchronization of cone receptivity and pollen shed is essential for the overall success of pollination. Variation in the duration of individual cone receptivity can be considered as one influence on the success of pollination in individual cones, because the pollen accumulates in a cone over a period of time.

From preliminary studies in 1971 experience was gained in assessing the different stages of cone receptivity and it was shown that second cycle cones could take six to seventeen days from emergence from their bud cataphylls<sup>x</sup> to receptivity. (Observations began after first cycle cones had closed and ceased before most second cycle cones reached the late receptive stage). Furthermore, duration of the stages before receptivity varied according to the relative advancement of the cone; it was longest in early-emerging cones and shortest in late-emerging cones. The duration of the receptive stage could only be measured for a few cones, for which it averaged ten days.

Daily records over a period of five weeks in 1972 also showed that the time taken for cones to reach receptivity was shorter for those emerging later (table 4:4). It was also shown that the duration of peak receptivity (the receptive stage) can vary between two and thirteen days, even within one clone (clone 274, figure 4:7).

Devel. group	Clone, tree, cycle	Developmental stage									90 no. cones.
		$\frac{1}{4}$ E	$\frac{1}{2}$ E	$\frac{3}{4}$ E	$\frac{7}{8}$ E	EFE	ER	R	LR	EC	
ER	274 T.1(1)							9.3	4.1	6.4	11
17th	274 T.3(1)						2.0	9.0	3.0	7.0	1
Aug.	55 T.1(1)							10.7	4.6	5.0	7
	89 T.6(1)						<u>2.0</u>	<u>10.0</u>	<u>3.0</u>	<u>6.0</u>	<u>1</u>
	Mean						2.0	9.8	4.2	5.9	20
	Pre-R, R, & post-R duration						NR	9.8		10.1	
$\frac{1}{2}$ E	274 T.1(1)			1.7	1.9	2.0	1.8	4.7	6.7	7.8	6
17th	274 T.3(1)			2.7	2.8	1.4	1.6	5.2	4.2	6.4	5
Aug.	55 T.1(1)			1.8	2.7	1.9	2.5	5.4	5.7	5.4	13
	89 T.6(1)			<u>3.0</u>	<u>1.7</u>	<u>1.4</u>	<u>1.9</u>	<u>2.5</u>	<u>5.8</u>	<u>5.6</u>	<u>11</u>
	Mean		*(2.5	2.6)	2.3	2.3	1.7	2.1	5.4	5.6	35
	Pre-R, R, post-R duration						ca13.5	5.4		12.0	
JE	274 T.1(1)	3.0	1.0	3.0	1.0	2.0	1.0	3.0	7.0	6+	1
17th	274 T.1(2)	2.4	3.2	1.4	1.6	1.6	2.2	7.0	3.9	NR	9
Aug.	55 T.1(1)	2.5	2.5	1.8	1.6	1.6	1.7	3.9	6.8	6.3	10
	89 T.1(1)	2.0	4.0	2.0	3.0	1.0	1.0	5.0	5.0	9.0	1
	89 T.6(1)	2.2	2.3	1.5	2.0	2.2	1.7	6.5	5.7	8.2	6
	372 T.2(1)	<u>5.0</u>	<u>1.0</u>	<u>2.0</u>	<u>3.0</u>	<u>0.0</u>	<u>2.0</u>	<u>10.0</u>	<u>2.0</u>	<u>7+</u>	<u>1</u>
	Mean	2.5	2.6	1.7	1.8	1.7	1.8	5.7	5.4	7.1	28
	Pre-R, R, post-R duration						12.1	5.7		12.5	
JE	274 T.1(2)	2.0	2.7	0.8	1.0	1.0	0.8	6.3	3.0	NR	3
24th	55 T.1(1)	2.0	3.0	2.0	1.0	1.0	1.0	3.0	6.0	8.0	1
Aug.	55 T.1(2)	2.0	2.9	1.4	0.9	1.3	3.1	5.8	4.6	5+	12
	89 T.6(2)	2.7	1.9	1.6	1.0	1.6	2.0	6.6	6.0	NR	7
	372 T.1(1)	2.2	2.0	2.0	1.4	1.4	2.2	11.0	6.0	NR	5
	372 T.2(2)	<u>1.7</u>	<u>2.5</u>	<u>1.5</u>	<u>1.2</u>	<u>1.7</u>	<u>2.5</u>	<u>8.0</u>	<u>5.3</u>	<u>NR</u>	<u>6</u>
	Mean	2.1	2.5	1.5	1.1	1.4	2.4	7.1	5.3	ca6	34
	Pre-R, R, post-R duration						11.0	7.1		ca11	
JE	55 T.1(1)	1.8	1.1	1.4	1.6	1.8	2.3	5.7	3.9	NR	9
29th	89 T.6(2)	1.8	1.3	1.5	1.5	1.3	1.5	6.0	6.3	NR	6
Aug.	372 T.1(1)	1.2	2.2	1.0	1.3	1.8	1.6	10.0	5+	NR	5
	372 T.2(2)	<u>2.0</u>	<u>0.7</u>	<u>1.0</u>	<u>1.3</u>	<u>3.0</u>	<u>2.0</u>	<u>10.3</u>	<u>NR</u>	<u>NR</u>	<u>3</u>
	Mean	1.7	1.3	1.3	1.4	1.8	1.9	7.3	4.9	NR	23
	Pre-R, R, post-R duration						9.4	7.3		NR	

\* estimated from the following group.

Table 4.4: Mean duration of each developmental stage shown by cones from different clones and cycles which emerged at intervals throughout the pollination period. The duration is in days.



In figure 4:7 the duration of cone receptivity for four of the observed clones is plotted against the date that each cone was first recorded at receptivity. Because the receptive stages extended through August and September, the actual dates were represented by their relationship to the first day of observation (16th August taken as day 0). Clone 19 was not observed daily because the trees were too frail to withstand frequent climbing and so the precise duration of cone receptivity could not be determined for this clone. Regression lines were drawn for the data and their significance tested using the t-test.

Significant negative relationships (0.1% level) were shown for first cycle cones of clones 274, 55 and 372, but not for first cycle cones of clone 89 or second cycle cones of clone 274 or 55. A significant (5% level) positive relationship was shown for second cycle cones of clone 89. Therefore, although there was a relationship between receptive duration and the date of receptivity in some cases, the relationships can differ between clones and between cycles within clones. Since the clones which reach first receptivity over much the same period (55 and 274) have similar regression equations, but show differences between first and second cycle cones, it seems that a factor associated with time, rather than a genetic factor, affects the relationship between receptive duration and the time of first receptivity. Possible factors are temperature, pollen density and weather conditions. However, the fact that the relationship in which the earliest cones (even in clone 89) remain open longest is not maintained throughout the pollination period means that rising temperatures cannot be accelerating cone development in a consistent way. Nor can the rise in pollen density in the air be associated with shorter receptive duration because cones receiving greater quantities of pollen after 30th August (shown in section 2 to be, in many cases, about thirteen times as much as that received prior to the 30th) generally remained open longer than cones which became receptive about the 30th August.

Furthermore, unpollinated, bagged cones do eventually close.

Closure may be developmentally determined, but the timing of closure is variable. To determine if weather conditions affect the timing of cone closure a second series of regression lines was calculated for the relationship between receptive duration and the number of days from first receptivity to rain (figure 4:8). Cones which became receptive on a wet day were plotted at co-ordinate 0 on the x axis. Variation along the y axis at this co-ordinate arose because there was a wet or humid period of four days (29th August to 1st September, inclusive) and many cones becoming receptive at the beginning of this period began to close before or just after it finished, while those which become receptive on the last day did not close until the following wet period (eight days later).

Nevertheless, significant positive relationships (0.1% level) were shown for first cycle cones of clones 55, 89 and 274 and second cycle cones of clone 89. A significant positive relationship (5% level) was shown for second cycle cones of clone 274, but not for second cycle cones of clone 55. The relationship for clone 372 became significant (1% level) when the data for cones emerging during the rainy period was excluded. The only group of cones which showed no relationship between receptive duration and the time of first receptivity to first rain was that of second cycle cones of clone 55. However, from figure 4:7, it can be seen that 75% of the sample became receptive between the 8th and 11th September. Although variation in their times of first receptivity was small, the period when most cones reached receptivity included one day (10th September) on which heavy rain fell, so that the length of time from first receptivity to rain was from 0 to 7 days, depending on whether the cones were recorded at first receptivity on the 10th or 11th of September. The shorter space of time between rainy days later in the pollination period means, also, that the x co-ordinates are close to-

gether and the relationship is more difficult to detect.

It is possible to check the weather effect on the pollination success of individual cones, taking the time of cone receptivity relative to pollen shed into account. This can be done by comparing the final pollination levels in a number of cones which were first receptive on recorded days (figure 4:9, clones 55 and 274, 1972). These clones were chosen because the data could also be compared with pollination levels in the same clones in 1971.

Unlike the cones harvested at receptivity (figure 4:2), the final pollen levels do not show a relationship with the times that the cones were receptive. This is probably because the duration of receptivity was so variable. Thus, in figure 4:9, the cone from clone 274, harvested on the 18th August received a very high final level of pollination, although it was shown in section 2 that at this time and up to the 30th August, receptive cones had received relatively small amounts of pollen. Records show that this well pollinated cone was receptive for thirteen days, so that the pollen accumulated throughout this period. Conversely, six of the twenty three cones from clones 55 and 274 had received less than two hundred pollen grains in the vicinity of forty ovules. Records show that each of these cones remained open from five to seven days before closing in the wet weather, either on the 29th August or the 19th September. Both of these wet periods occurred outside the period when higher levels of pollen were trapped in receptive cones (4th to 16th September). Therefore, the combination of a shorter receptive period and its occurrence before, or after the period when pollen shed is at its peak, does affect the pollination success in individual cones. In the subsequent section it will be shown that the quantity of pollen trapped must be greater than one hundred and sixty to three hundred grains if the micropyles are to be filled to capacity.

A comparison of figures 4:7 and 4:8 shows that the relationships

between receptive duration and the time of first receptivity may be really determined by the pattern of wet days throughout the pollination period. The lack of a relationship between duration and first receptivity date in first cycle cones of clone 89 and second cycle cones of clone 55 could arise because the wet period occurred in the middle of clonal receptivity, while in clones 55, 274 and 372 it occurred towards the end of first cycle receptivity. Conversely, the slight positive relationship shown in figure 4:7 by second cycle cones of clone 89 might have arisen because the wet period was towards the beginning of second cycle cone receptivity and cones which became receptive just before or during the wet days closed only a few days later.

The influence of wet periods on general receptivity in the stand can be extrapolated from the data from the five observation clones (figure 4:1). Because the percentage of receptive clones declined after the 29th August, the number of cones closing must have exceeded the number becoming receptive. The first decline in numbers of receptive cones coincided with the first period of wet days. The percentage of receptive cones dropped for a second time after the 10th September (the second period of heavy rain), but continued to drop because the proportion of cones in prereceptive stages had diminished to low levels by this time.

The appearance (visual) of receptive cones in the rain suggests that the tissues have become swollen with water, even though the surface of the ovuliferous scales seems waxy and water repellant. Under conditions of rain the moisture deficit in the tree is decreased and this may trigger cone closure.

Subjective judgement of cone receptivity on the basis of visual appearance could accentuate the number of cones first recorded in the late receptive stage in wet weather and give rise to the relationship shown between cone closure and wet weather (figure 4:8). Nevertheless, subsequent records for these cones are generally consistent with the

earlier assessments and the late receptive cones do not revert to receptive stages in fine weather following rain.

(6) Limited Pollen Arrival and Clonal Differences in Micropylar Capacity as Factors affecting the Success of Ovule Pollination.

From the figures 4:10 and 4:11, which show the amounts of pollen trapped in a number of cones, it can be seen that some cones at Whaka, but very few at Kaingaroa, trapped less than two hundred and thirty pollen grains in the vicinity of forty ovules. (This number is taken as the mean of the range one hundred and sixty to three hundred pollen grains necessary for filling the micropyles. The reason will be given shortly.) Of the cones from 1971 and 1973 from Whaka, 90% and 66%, respectively, had trapped fewer than two hundred and thirty grains, but in 1972 only 20% of the cones at Whaka had trapped pollen in amounts as low as this. Thus, the proportion of cones which are successfully pollinated may be high or low depending on the pollination year and site.

However, most cones had received a mean of at least one grain per ovule ( more than forty grains trapped in the vicinity of forty ovules). Inadequately pollinated cones, receiving less than this did occur at Whaka in 1971 and 1973 (20% and 10% of the sample, respectively).

Therefore, in years in which the levels of pollen trapped is lower, some cones will be poorly pollinated, but even in these years the proportion of inadequately pollinated ovules is not really high. In good years for pollen shed at Kaingaroa, the majority of cones trapped pollen far in excess of that required for successful pollination.

It has been shown that the quantity of pollen received by a cone depends on the duration of receptivity for that cone and on the amount of pollen being shed during that period. However, only a proportion of pollen arriving in the cone can be accommodated in its micro-

pyles. Only this proportion is able to continue growth and can potentially fertilize the egg cells. The amount of pollen which reaches the micropyles is therefore of greatest significance for the final success of pollination, but it is dependant on sufficient quantities of pollen arriving in the vicinity of the ovules.

Figures 4:10 and 4:11 show that the amount of pollen accommodated in the micropyles of forty ovules increases as the amount of pollen in the cone increases, up to a point where usually between a hundred and sixty and three hundred grains are received by forty ovules in the cone. It is not possible to put a more precise figure because, even when pollen arriving is not limiting, there is considerable variation in the pollen which reaches the micropyles in different cones. When more than a hundred and sixty to three hundred pollen grains are received by cones the number held in the micropyle is apparently limited by another factor for the relationship between pollen in the micropyles and pollen trapped by the cone is discontinued.

In eighty six cones which had trapped more than a hundred and sixty grains, the mean number of grains in the micropyles did not exceed a hundred and sixty. Dissections and sections of thousands of ovules showed that micropyles containing more than four pollen grains are comparatively rare, except in a few cones of clone 55, and the micropyles seem to be full to capacity with four grains. Sarvas (1962, 1967) considered that the size of the micropyle of P.sylvestris limited the amount of pollen it could contain and this is also true of P.radiata. When small amounts of pollen are trapped by a cone the amount of pollen entering the micropyles is limited, but when more than a hundred and sixty to three hundred grains are available to the forty ovules, the size of the micropyle becomes limiting and pollination is very successful.

The amount of pollen necessary for successful pollination has been given as a range because cones vary in the amount of pollen they can accommodate in their micropyles. Clone 19 cones may have only a few

ovules with micropyles large enough to contain four pollen grains and many have only two grains. The arrival of a hundred and sixty grains in the vicinity of forty ovules is, in this case, probably enough to ensure that all ovules are filled to capacity. Some clone 55 cones have a large proportion of ovules with room for four pollen grains, so in these cones three hundred or so grains must be trapped by the cone to ensure that all micropyles are filled.

A statistical study of the amount of pollen accommodated in the micropyles of the two clones, 19 and 55 was carried out on cones harvested for morphological studies. Since counts were made from sections, which are time consuming to prepare and examine, data was obtained for thirty ovules. An analysis of variance and F tests were carried out on the means shown in table 4:5.

Clone	Year					Mean
	1967	1968	1969	1970	1971	
55	104	97	108	94	67	94
19	39	56	62	50	58	53
Mean	72	77	85	72	63	
LSD	5% = 17, 1% = 23					

Table 4:5 The number of pollen grains trapped in the micropyles of thirty ovules per cone. (means of five cones).

Analysis of variance (appendix 4) showed that there is a highly significant difference (<sup>0</sup>1% level) between the amount of pollen contained in the micropyles of the two clones. There was also a significant (1% level) between the years and a significant (0.1% level) clone-year interaction was shown. L.S.Ds (table 4:5) showed a significant (1% level) clonal difference in all years but 1971 and also showed that 1971 was the only year in which the pollen in the micropyles was sign-

ificantly different from all others. However, the difference was shown only by clone 55 cones. The amount of pollen in the clone 55 micropyles was quite anomalous in 1971 and apparently contributed to the significant clone-year interaction. In section 4 it was shown that the quantity of pollen trapped was less in clone 55 at Kaingaroa in 1971 than in other years for the same clone and site. Even so, three of five cones received more than two hundred and eighty grains in the vicinity of forty ovules. It appears, then, that the micropylar capacity of clone 55 was reduced in 1971. Sweet and Thulin (1972) report that the incidence of graft incompatibility at this seed orchard increased at this time and that 61% of clone 55 trees and 21% of clone 19 trees showed symptoms of graft incompatibility. It is possible that, of the cones of clone 55, more were debilitated and the size of the ovules affected as a consequence. This same effect of graft incompatibility could have resulted in reduced pollen availability at the site.

#### (7) Variation in the Pollination of Ovules within Cones

Up to this point pollination in P. radiata has been considered in terms of pollen levels in samples of cones because the factors affecting the amount of pollen available to ovules are those which affect the whole cone. (i.e. cone receptive duration and its coincidence with pollen shed and weather conditions.) But, the number and variation of pollen grains in individual ovules within cones is of concern to the geneticist and tree breeder. It is not, however, possible to give a mean number of pollen grains, accompanied by a standard deviation because the distribution is skewed. The upper limit, depending on the clone, is determined by the size of the micropylar chamber, and, as long as the pollen supply is not limiting, large numbers of micropyles could receive the upper limit of pollen. Therefore, the variation in the percentage of ovules having 0, 1, 2.....pollen grains is shown for a selection of clones and cones from P. radiata (table 4:6).



Year	Site	Clone	Cone	0	1	2	3	4	5	6	no. p. grains trapped per 40 ovules
1970	Kai.	55	1	0.0	11.5	3.8	38.5	26.9	19.2		748
			2	3.4	13.8	20.7	41.4	17.2	3.4		756
			3	0.0	3.3	10.0	40.0	40.0	6.7		800
			4	0.0	7.1	14.3	28.6	39.3	10.7		820
			5	0.0	11.1	11.1	38.9	33.3	5.6		828
			6	3.7	7.4	18.5	40.7	26.0	3.7		1080
1970	Kai.	19	1	40.6	39.1	12.2	7.3				70
			2	6.7	23.3	40.0	30.0				324
			3	0.0	20.0	70.0	10.0				364
			4	15.2	21.2	39.5	24.2				576
			5	6.9	31.0	31.0	17.2	10.3	3.4		765
			6	6.7	40.0	33.3	20.0				82
1971	Kai.	55	1	7.7	26.9	27.0	30.0	7.2			136
			2	3.1	25.0	43.8	25.0	3.1			140
			3	0.0	29.2	29.2	29.2	12.5			272
			4	16.0	44.0	32.0	4.0	4.0			304
			5	3.4	6.9	24.1	62.1	3.4			312
1971	Kai.	19	1	3.2	9.7	45.2	38.7	3.2			256
			2	3.7	0.0	59.3	29.6	7.4			288
			3	2.8	8.3	27.8	50.0	11.1			304
			4	6.9	3.4	31.0	51.7	6.9			320
			5	0.0	4.4	8.7	60.9	21.7	4.4		320

Table 4:6 Percentage distribution for individual cones of ovules accommodating 0, 1, 2... pollen grains in the micro-pyle. Table continued on page 100 and 101.

Year	Site	Clone	Cone	0	1	2	3	4	5	6	no. p. grains trapped per 40 ovules
1971	Whaka.	55	1	12.8	18.0	20.5	41.0	5.1	2.5		148
			2	0.0	8.0	32.0	40.0	16.0	4.0		148
			3	0.0	3.7	29.6	25.9	37.0	3.7		180
			4	0.0	0.0	16.0	32.0	36.0	16.0		184
			5	0.0	0.0	13.8	24.1	24.1	37.9		184
1971	Whaka.	19	1	17.7	23.5	50.0	5.9	2.9			68
			2	34.2	39.5	21.1	5.1				72
			3	10.3	23.1	51.3	15.4				148
			4	5.1	35.9	38.5	20.5				192
			5	0.0	29.4	58.8	11.8				240
1971	Whaka.	274	1	48.0	48.0	4.0					92
			2	23.1	50.0	23.1	3.8				96
			3	6.1	30.3	42.4	21.2				196
1971	Whaka.	372	1	2.9	11.8	50.0	32.3	2.9			156
			2	0.0	92.6	7.4					212
			3	0.0	13.0	21.7	39.1	26.1			216
			4	0.0	34.4	31.2	31.2	3.1			224
			5	0.0	0.0	34.5	62.1	3.4			408
1972	Whaka.	55	1	2.5	20.0	40.0	32.5	5.0			260
			2	2.5	2.5	5.0	60.0	27.5	2.5		320
			3	2.5	10.0	17.5	37.5	30.0	0.0	2.5	360
			4	0.0	0.0	16.7	26.7	36.7	16.7	3.3	444
			5	0.0	0.0	20.7	48.3	27.6	3.4		448
			6	3.7	7.4	25.9	48.2	14.8			560

Table 4:6 continued.

Year	Site	Clone	Cone	0	1	2	3	4	5	6	no. p. grains trapped per 40 ovules
1972	Whaka.	274	1	40.0	16.7	23.3	13.3	3.3	3.3		60
			2	10.0	20.0	45.0	25.0				120
			3	5.0	30.0	50.0	15.0				224
			4	5.0	25.0	22.5	37.5	7.5	2.5		260
			5	0.0	27.6	51.7	20.7				308
			6	0.0	15.0	30.0	45.0	10.0			390
			7	0.0	5.0	22.5	32.5	32.5	7.5		464
			8	0.0	3.3	16.7	53.3	23.3	3.3		752
1973	Whaka.	55	1	6.7	23.3	36.7	30.0	3.3			164
			2	3.3	30.0	33.3	26.7	6.7			232
			3	0.0	3.3	20.0	23.3	36.7	13.3	3.3	236
			4	0.0	3.3	20.0	53.3	20.0	3.3		288
			5	0.0	3.3	10.0	33.3	40.0	10.0	3.3	328
			6	3.3	3.3	26.7	43.3	23.3			340
1973	Whaka.	19	1	36.7	43.3	10.0	6.7	3.3			40
			2	23.3	36.7	20.0	13.3	3.3	3.3		68
			3	16.7	13.3	30.0	30.0	10.0			124
			4	6.7	6.7	26.7	40.0	20.0			136
			5	6.7	6.7	6.7	46.7	26.7	6.7		212

Table 4:6 continued.

Comparisons of the data for each cone show, again, the factors found in section 6 which affect the amount of pollen in the micropyles. (Limited pollen supply and clonal differences in the micropylar capacity.) It also shows the variation within cones. The effect of limited pollen can be seen in the data from cone 1, clone 274 from Whaka in 1972. A few ovules did contain four and five pollen grains and probably more had the same capacity, but there were no pollen grains available, because there were only sixty grains available to forty ovules.

Another factor affecting the amount of pollen which can potentially fertilize egg cells, must arise from a difference in micropylar size between cones within the same clone. This conclusion is reached by studying the data from clones 2 to 6, clone 55 at Whaka in 1972. The number of pollen grains trapped by these cones would be expected to be enough to fill almost all the micropyles to capacity. Thirty percent or more of the ovules in cones 2, 3, 4 and 5 contained more than three pollen grains, but only about fifteen percent of ovules in cone 6 did. Cone 6 was a single cone, borne on a small first order branch towards the top of the tree and was possibly in a less advantageous position for attracting nutrients than cones 2 to 5 which were borne on larger branches, lower in the tree.

Since the number of pollen grains accommodated by ovules in well pollinated cones is variable, it is of interest to find if the variation is affected by the position of the ovule in the cone, an observation made by Bramlett (1973). Ovules were dissected from specific zones (tip, middle and low regions of the zone of normal ovules in the cone). Five cones of clone 19 (1967) and one of clone 55 (1967) were dissected as well as two of clone 19 (1970). It was not possible to sample equal numbers of ovules in each zone because the number of ovules in the middle and low zones is greater than the number at the tip owing to the shape of the cone. The proportion of ovules from each zone accommodating 0, 1, 2... pollen grains is shown in figure 4:12.

The pollen trapped in the 1967 cones could not be counted because the ovules were dissected from year old cones, but it is possible that the amount trapped in cone 1 was low. However, even when cones are well pollinated, the ovules in the low zone usually accommodate 0 to 2 grains. Ovules from the middle zone make up a higher percentage of those containing more than two grains, while ovules in the tip zone show the range of variation, 0 to 5 grains.

In all cones it is noticeable that the lowermost ovules bordering the zone of rudimentary ovules look smaller and often contain fewer pollen grains than ovules in the rest of the cone. Average measurements of ovule length from ovules dissected and sectioned from six cones were all smaller in the lowest ovules. The magnitude of the difference depended on the stage of ovule development; it was 0.1 to 0.2mm in year old cones with a mean length of 2.5mm and at fertilization, three months later, it was 0.5mm when seed length in the rest of the cone ranged from 5.5 to 6.0mm. At the time of pollination the difference in size between the lowest ovules and those in the rest of the cone would be very small, because the ovules themselves were only 0.3 to 0.4mm, but was possibly sufficient to exclude pollen grains when there<sup>w</sup>ere already one or two grains in the micropyle.

Because the proportion of smaller ovules in the ovule bearing zone is not large and because sampling in both dissected and sectioned material would include similar proportions of such ovules in all the cones, their inclusion probably would not alter the estimates of pollination success.

#### (8) Artificial Pollination

The previous sections have given a picture of the success of pollination of cones and ovules in natural conditions. Tree breeders are concerned that the techniques of applying pollen to bagged cones for crossing specific genotypes result in successful pollination of the cone.

In 1970 cones of clone 89 were artificially pollinated on the 20th August. The amount of pollen in cones, harvested when they were closing, was generally less than a hundred grains per forty ovules and only six of the cones (sample size, twelve) trapped enough grains to supply most ovules with at least one grain. The stage of receptivity of the cones at the time they were pollinated ( in humid weather) was not recorded, although it varied between cones in the same bag and between all the cones pollinated on the one day. The presence of prereceptive and postreceptive cones in the bag could account for the cones with low levels of pollen. However, it appears that in general, the bagged cones trapped very little pollen, perhaps because insufficient pollen was applied or because the pollen adhered to the damp surfaces of the bag, shoot and outer cone and did not circulate in the bag. It is also possible that receptive bagged cones, like unbagged cones, begin to close in wet weather, because of alteration in the moisture stress of the tree.

In 1971 the experiment was repeated: the stage of receptivity of each bagged cone was recorded, excess pollen was applied and cones were harvested when they had closed. In clone 89 pollination with dead pollen was carried out on 17th August at the beginning of a week of fine weather. Pollination of the same cones on the 23rd August was with living pollen. This meant that the pollen which had arrived on different days could be identified, depending on whether it had germinated or not. (In chapter VI it will be shown that pollen inviability is low in naturally pollinated cones and in cones artificially pollinated with untreated pollen.) Cones which were first pollinated at receptivity in fine weather had full micropyles and all the included pollen was dead. On the other hand, cones which were first pollinated in stages between  $\frac{1}{2}$ E and  $\frac{7}{8}$  E were inadequately pollinated with thirty to sixty grains per forty ovules, and both dead and living pollen were identified in their micropyles. This suggests that pollen arriving prior to receptivity can enter the cone in limited amounts, but, as described in chapter III, the

best time to pollinate cones is when they are receptive, during fine weather. One way analysis of variance and an F test showed that the amount of pollen in the micropyles from ovules in 1971, pollinated at ER and R was significantly (1% level) higher than the amount in 1970. Thus, both poor and excellent pollination can be achieved by artificial techniques.

It has been shown that, in conditions of natural pollination, the success of pollination can vary from year to year, according to the amount of pollen available at the time of cone receptivity and to the duration of receptivity in individual cones. With artificial pollination excess pollen can be applied to fully receptive cones for a short time so that there should be no difference in the success of pollination within and between years.

In 1971, although cones of clone 89 received an average of a hundred and nine grains inside forty micropyles, cones of clone 372, pollinated at ER and R (on 23rd and 24th August) received an average of only sixty two grains inside their micropyles. These differences were significant (5% level) and could be due to either clonal differences in micropylar capacity or to different conditions operating on the days of artificial pollination, but when clone 372 (1971) micropylar pollination levels achieved by artificial pollination were compared with equivalent naturally pollinated cones, the analysis of variance did not detect any difference. This suggests that neither the quantity of pollen, nor the difference in the environment of the bagged cones affected the amount of pollen in the micropyles, but that the capacity of the micropyle was limiting pollen quantity in both cases.

In 1972 the same treatment was given to a larger sample of cones of clone 372. Receptive bagged cones were artificially pollinated on the 29th and 31st August in humid weather. (Results of artificial and natural pollination for clone 372 (1972) have been given in figures 3:9 to 3:11.) Analysis of variance showed that the micropylar pollination

levels were significantly higher ( 5% level) in 1972 than they were in 1971, but, as in 1971, the amount of pollen in the micropyles of artificially pollinated cones was not significantly different from the amount in naturally pollinated cones.

The amount of pollen trapped in the cones was also measured in 1972. In artificially pollinated cones the mean number of grains trapped in the vicinity of forty ovules was 856 grains and this was significantly higher (<sup>0</sup>1% level) than the mean number in naturally pollinated cones (214), harvested at receptive and post receptive stages.

The between year and clone difference in the amount of pollen in the micropyles was not related to the technique of artificial pollination, provided the cones were receptive at the time of pollen application. Instead, it seemed to be related to variations in the micropylar capacity in different cones.

It has been noticed (M.P. Bollmann, Forest Research Institute, pers. comm., 1971) that cone development is more rapid in bagged cones. The daily records for bagged cones could be compared with those for eight unbagged cones, which emerged from their cone bud cataphylls at the same time as the bagged cones did. The average duration of ~~receptivity~~ <sup>in the receptive period</sup> for each stage of development is given in table 4:7.

	Developmental stage										Time to EC	Time to R	no. cones
	JE	$\frac{1}{4}$ E	$\frac{1}{2}$ E	$\frac{3}{4}$ E	7/8	EFE	ER	R	LR	EC			
B	2.3	1.8	1.1	1.4	1.1	1.2	2.4	8.2	3.4	6.9	22.9	11.3	25
UB	4.1	2.6	2.1	1.8	1.9	1.4	2.1	9.3	5.5	NR	30.8	16.0	8

Table 4:7      Mean duration (in days) of each stage of receptive development in bagged (B) and unbagged (UB) first cycle cones of clone 372.



Table 4:7 shows that, although the mean receptive duration is shorter by only one day (in bagged cones), the mean time taken to reach receptivity is reduced by four to five days. Reduction in the time taken to reach receptivity is relatively greater than the reduction at the receptive stage.

#### IV. DISCUSSION

##### (1) Duration of the Pollination Period

The pollination period is taken to be the time when pollen is being shed and / or when female cones are receptive. Usually pollen shed and cone receptivity are concurrent, but there have been reports that cone receptivity can last longer than pollen shed (Nekrasova, 1970) or that pollen shed is delayed by wet weather and cone receptivity is not (Sarvas, 1962). From observation in a P.radiata clonal area in two years, it appears that the proportion of cones receptive when pollen shed ceases is low, and the amount of pollen being shed when few cones are receptive is also low. This needs to be studied in detail in other years and on other sites. The type of data given by Sarvas (1967) for P.sylvestris, showing the temperature sum (measured in degree days above 5°C) necessary for the commencement of cone receptivity and pollen shed might also be obtained for P.radiata. Relationships between threshold temperature sums for cone receptivity and pollen shed might be shown.

Data for P.radiata pollen shed in Australia (Millet, 1944; Fielding, 1957) can be compared with the duration of pollen shed at Whaka in 1972. The Whaka results fall into the range given by these authors, so it can be concluded that pollination in P.radiata covers a longer period than any other species mentioned in the literature. The polycyclic nature of P.radiata may have contributed to the evolution of an extended pollination period. However, it was shown in section 2 (observations and results) that the onset of receptivity in first and second cycle cones of clone 274 occurred before that of first cycle

cones of clone 372. Therefore, the long duration of general receptivity is not due to the polycyclic shoot structure alone, but also to genetic variation in the onset of clonal receptivity. Observations on clonal areas of other species of Pinus might show a slightly longer pollination period than is at present recorded for those species.

Whatever the selection pressure giving rise to a particularly long pollination period, the result is that unfavorable weather conditions would not affect all of the cones and pollen in the stand or clonal area. Therefore, in any year most or at least some of the cones should be well pollinated. In some years early or late cones might be inadequately pollinated, but their proportion relative to the total number of cones would be small.

## (2) Effect of Weather Conditions on the Success of Pollination

The correlation between the onset of cone closure and wet weather has not been noted in the past, possibly because the progress of individual cone development has not been observed. Furthermore, if wet conditions occur at frequent intervals over a pollination period, the duration of receptivity would be shorter in all cones and also less variable, so that the relationship would be difficult to detect.

Wet conditions may not be the sole cause of cone closure, but may need to be associated with physiological conditions present in the cone after at least one or two day's receptivity. The duration of cone receptivity in cones which become receptive close to a wet day is more variable than in cones which become receptive during a dry period. However, there is evidence that most receptive cones become swollen in wet weather: the passages between their scales are constricted and from that time they continue to close. This means that cone closure is triggered when there is little available pollen in the air.

If wet weather affects cone closure by altering the degree of water stress in the tree, it should affect bagged cones as well as unbagged cones, even though water may not be in direct contact with the

cone surface. The data in table 4:7 supplies information on this, but because the number of cones observed at LR (late receptivity) was low in both samples, it cannot be taken as proof. Cone development up to receptivity was five days faster in bagged cones than in unbagged cones which took sixteen days to reach receptivity, but the duration of receptivity, itself, was only one day shorter than the nine days of receptivity for the unbagged cones. However, because bagged<sup>cones</sup> were receptive five days earlier, they closed with the rainy period around the 10th September, whereas the unbagged cones closed with the wet days of the 16th September. These observations fit the idea that bagged cones are affected by wet weather in the same way as unbagged cones are. A dry period would mean that bagged cones remained receptive longer and there would be more time available for artificial pollinations. Brown (1973) showed that seed production in bagged cones paralleled the variation in seed production in unbagged cones from year to year. Possibly wet conditions reduced the receptive time for P. sylvestris cones in some years so that the cones were not so wide open when pollen was present in the air, or applied in the bag. More detailed research along the lines of this study could provide useful information.

### (3) The Quantity of Pollen Trapped by Cones

It has been shown that there is considerable within tree variation in the amount of pollen arriving in cones. Cones with little pollen in them can close during a wet period after a short receptive duration. The position of the cone on the tree and its orientation on the shoot might produce some variation in the pollen trapped. Usually cones are at the extremities of the longest branches and are clear of the needle<sup>s</sup> which are not fully elongated at the time of cone receptivity. The cones protrude into free airspace and the branch is moved by the wind so that pollen borne on air currents would have unhindered access to most cones. The results of this study suggest that pollen arriving in cones is limited mainly by weather conditions and the developmental stage of the

cone rather than by the quantity of pollen shed during the pollination period. Even so, the majority of cones trapped more than a hundred and forty grains per forty ovules (i.e. enough to pollinate most ovules with one or more grains in the micropyle, table 4:6) Many cones receptive at a variety of times within the pollination period trapped amounts greatly in excess of this.

Pollen levels at Kaingaroa were generally much higher than they were at Whaka, although direct comparison was possible in 1971 only. It would be expected that Kaingaroa, an established seed orchard of greater area would produce more pollen than Whaka, a young clonal area. Despite a buffer region of other species, it is also possible that pollen could arrive from other regions of Kaingaroa forest because the area covered there by P.radiata is so great and huge clouds of pollen can sometimes be seen moving over the forest in the spring (G.B.Sweet, pers. comm.)

As long as site quality is not limiting pollen production, it is possible that a trend towards increase in pollen shed is occurring at Whaka as the clonal area matures, but good conditions for pollen production may result in variation between consecutive years. But, even at Whaka pollen arrived in cones in quantities which were sufficient to supply most ovules with at least one grain.

#### (4) The Quantity of Pollen Trapped in Ovule Micropyles

Although only one pollen grain need enter the micropyle for the ovule to continue development, two or more grains provide a range of genotypes in the resulting embryos on which selection can operate. However, it appears that P.radiata clones have a different capacity for accommodating pollen grains in their micropyles. Even in years when large amounts of pollen are trapped, clone 19 cones rarely contain more than 2.5 grains in the mean micropyle, but in the same years clone 55 micropyles usually contained a mean of more than three grains. Other clones which were examined gave means between or comparable to these. Means of two to three pollen grains with five or six at the

most are comparable to the amount of pollen found in the micropyles of P.virginiana (Thomas, 1951) and P.sylvestris (Brown, 1970).

As Bramlett (1973) suggested, the position of an ovule within a cone affects the level of its pollination. In P.radiata this does not appear to be due to a reduced ability for the low regions to trap pollen, because the lowest normal ovules occur at the widest part of the cone and plenty of pollen can be seen on them. It is more likely that the capacity of the micropyle in the lowest of the normal ovules is generally smaller and possibly limits the amount of pollen which can be accommodated. Furthermore, variation in the size of ovules (and their micropyles) in cones on different parts of the tree probably do not affect the success of fertilization seriously, though they may reduce, for selection, the genotypes available. The relationship of the number of pollen grains to the number of archegonia will be discussed in Chapter VI.

Results from artificial pollination and natural pollination of clone 372 suggest that the micropylar capacity within clones can vary between years, according to the state of the tree. The difference might be determined by temperature or other climatic effects at the time of prereceptive ovule development. Alternatively, the mean ovule size and micropylar capacity might be related to the nutrient status of the tree and, if so, it could be influenced by the application of fertilizers. Stimulation of cone production in seed orchards has been shown to result from application of fertilizers at the time of cone initiation (R.C.Kellison, pers. comm.). It is possible that cone size from the time of emergence is also increased.

However, when limited pollen is trapped by a cone, a large proportion of ovules may remain unpollinated and will abort during the subsequent development of the cone. A mean of one or even two grains trapped for every ovule is not sufficient to pollinate all ovules in a cone. In P.sylvestris the regression of the percentage of non-pollinated ovules

on the effective pollen catch was very similar to the regression of the percentage of aborted ovules on effective pollen catch (Sarvas, 1962). But, usually lack of pollination is not a major cause of seed failure in P. radiata. The frequency of unpollinated ovules in P. radiata will be considered in chapter VI.

#### (5) Statistical Analysis

For this study only small numbers of cones were available from Kaingaroa in sizes suitable for microscopic sectioning of whole cones. The time that could be spent on dissecting and counting pollen was also limited. Since the samples were generally small and the variation considerable, it is possible that in some comparisons real differences between years, clones and sites might not have been detected. Nevertheless, from the significant differences that were shown, it can be seen that pollen arriving in cones can be affected by the pollination year and site, and that the amount of pollen capable of being accommodated in the micropyle is genetically determined, but may also be affected by year influences.

## CHAPTER V

## THE MORPHOLOGY OF DEVELOPING OVULES AND SEEDS SUBSEQUENT TO CONE CLOSURE

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## IV. DISCUSSION



## CHAPTER V

## THE MORPHOLOGY OF DEVELOPING OVULES AND SEEDS SUBSEQUENT TO CONE CLOSURE

## I. INTRODUCTION

Morphological information on the life history of pines has accumulated since the 19th Century, but has not generally been related to the success of seed production. The process of fertilization has received particular attention (Dixon, 1894; Coulter, 1897; Blackman, 1898; Chamberlain, 1899; Haupt, 1941; McWilliam and Mergen, 1958; Camefort, 1965a, 1969). Embryology has been studied in detail by Buchholtz (1918, 1931), Doyle (1963) and Berlyn and Passof (1965). The structure of the mature embryo was described by Spurr (1941). Authors of complete life history studies were given in chapter II.

From the literature it seems that pines are all very alike in their reproductive development. Several accounts of reproduction in conifers, generally, show that the developmental cycle and structures in Pinus are comparable in many other conifer genera. (Maheshwari and Singh, 1966; Konar and Oberoi, 1969; Singh and Johri, 1972). The life history can be separated into a series of stages. Stages 1 and 2 (cone initiation and pollination) have been described in previous chapters. Stage 3 (pollen germination), stage 4 (megaspore formation), stage 5 (prothallial and archegonial growth) and stage 6 (fertilization and embryogeny) will be considered as separate sections of this chapter. Within each stage the development of the various tissues will be followed separately.

Although these stages have been shown in all the species of Pinus studied, the times taken to reach each stage varies between species (Table 5:1). With the present day economic importance of pines and the resultant interest in promoting seed production in genetically selected trees, it is important to establish the timing of morphological changes in species and localities used for seed production.

Species	Author and date	Stages	Duration	no. of archeg.
<u>P. strobilus</u>	Ferguson, 1904	Polln.-meiosis	26 dys.	1-5
		Polln.-fert.	12 mnt.	
<u>P. rigida</u>	" "	Polln.-meiosis	9 dys.	1-5
<u>P. resinosa</u>	" "			1-5
<u>P. austriaca</u>	" "			5
<u>P. montana var. uncinata</u>	" "			5-9
<u>P. roxburghii</u>	Sethi, 1928	Polln.-meiosis	14 dys.	2-7
		Polln.-fert.	13 mnt.	
<u>P. strobilus</u>	Chamberlain, 1935	Polln.-fert.	12½ mnt.	
<u>P. larico</u>	Johansen, 1940	Polln.-fert.	13 mnt.	2-6
<u>P. lambertiana</u>	Haupt, 1941	Polln.-fert.	12 mnt.	5
<u>P. monophylla</u>	" "	Polln.-fert.	12 mnt.	3
<u>P. ponderosa</u>	Roeser, 1941	Polln.-fert.	13 mnt.	
<u>P. virginiana</u>	Thomas, 1951	Polln.-meiosis	7-22 dys.	2-3
		Polln.-fert.	13½ mnt.	
<u>P. nigra</u>	McWilliam & Mergen, 1958	Polln.-fert.	12-13 mnt.	
<u>P. roxburghii</u>	Konar, 1960	Polln.-meiosis	0 dys.	2-4
		Polln.-fert.	13 mnt.	
<u>P. wallichiana</u>	Konar, 1960	Polln.-meiosis	0 dys.	1-2
		Polln.-fert.	11½ mnt.	
<u>P. gerardiana</u>	Konar, 1962	Polln.-meiosis	0-2 wks.	1-4
		Polln.-fert.	11½-12 mnt.	
<u>P. sylvestris</u>	Sarvas, 1962	Polln.-meiosis	3 wks.	1-4
		Polln.-fert.	12½-13 mnt.	
<u>P. siberica</u>	Nekrasova, 1970	Polln.-fert.	13 mnt.	1-3

Table 5:1 Duration of developmental stages in days (dys.), weeks (wks.) or months (mnt.) and the numbers of archegonia for species reported in the literature.

A knowledge of normal ovule development is useful for providing a background to the study of ovule and embryo failure, considered in chapter VI.

Rather than report the detailed observations of other authors in an introductory section, they will be discussed in the appropriate section of the observations and results, to avoid repetition. To complement the text a detailed series of photographs are presented in volume II.

## II. MATERIALS AND METHODS

### (1) Material

Most material used in the developmental study was harvested by the Forest Research Institute during the period from mid July, 1968 to April, 1971. It came from clones 19 and 55 from the site at Kaingaroa, described in chapter II. Additional material from clones 7 and 121 (also from Kaingaroa) was harvested for intended study of the development of unpollinated cones. However, these cones remained open in their bags for an extended period and were naturally pollinated when the bags were removed too early (about three weeks after the cones were estimated as being receptive). Because clones 7 and 121 were harvested at weekly intervals, they provide more exact information on the timing of meiosis relative to pollination. Other harvests were at fortnightly intervals, except for those from January to May (1969), which were at monthly intervals.

A pilot experiment to determine the effect of self pollination on the germination of pollen was carried out at Whakarewarewa. A sample of cones of clone 89 were pollinated artificially, half with pollen taken from clone 89 trees and half with a mixture of pollen from a large number of clones. After five days one cone from each treatment was harvested and fixed daily. It was later sectioned and the amount of pollen which

had germinated was counted in each. This material gave information on the time of pollen germination after pollen arrival and on the size of ovules in the weeks following pollination. The experiment was not followed up because the effects of self pollination on germination were not marked and would require more time than was available to get data which could be analysed statistically.

Some material from cones of clone 372 was harvested from Kain-garoa (1970), six weeks after receptivity. Data on meiosis times in this material is therefore included.

## (2) Fixation

Material harvested by the Forest Research Institute was fixed in F.A.A. However, the effects of fixation with the following solutions were investigated using ovules from a pine tree in Christchurch (P. radiata).

- i. pure acrolein
- ii gluteraldehyde in 10% ethanol
- iii Craft's solution
- iv osmium tetroxide

After fixation all were dehydrated in an ethanol-T.B.A. series, infiltrated in wax and embedded in paraplast. They were stained with safranin and fast green. Photographs showing the difference in the resultant appearance of archegonia, harvested at the same time can be seen in photographs 29C, D, and E. Photograph 29B shows archegonia fixed in F.A.A. by the usual method. A photograph of material fixed in osmium tetroxide is not included, because it stained the cell contents densely black and little detail could be seen. Best fixation was achieved with acrolein fixation of ovules dissected in the field.

## (3) Embedding

The embedding medium used for most material was paraplast, but glycol methacrylate, following acrolein - gluteraldehyde fixation was

also used (Feder and O'Brien, 1968). The resulting cell preservation was excellent, but the time taken to cut sections (using glass knives) and the folding, which occurred with large sections, limited its use.

#### (4) Staining

Safranin and fast green provided a satisfactory general purpose stain for F.A.A. fixed, paraplast embedded material. A selection of other stains were used on a small number of ovules to find their effects.

Tannic acid- iron alum- safranin- orange G possibly provided better results for black and white photography. The appearance of material stained with alcoholic haematoxylin and orange G was very similar.

Wax embedded material, cut and carried through a xylol- ethanol series, was stained in 2% aqueous solution of toluidine blue, for one hour, and, after washing, with 2% aqueous acid fuchsin for ten minutes. Cell walls and nuclei stained bright blue, but the cell wall of the multinucleate megaspore was mauve.

Both toluidine blue and periodic acid- Schiff's reagent were used to stain material embedded in glycol methacrylate (appendix 5), but haematoxylin or acid fuchsin were also successful. P.A.S. stained starch grains a deep purple and showed up the reticulate nature of the egg cell wall, which was also stained purple.

#### (5) Dissection of Embryos

Young embryo systems were dissected from seeds in 0.25 M phosphate buffer at pH 7.2 and then were fixed in F.A.A. They were dehydrated and infiltrated according to the schedule given for a gradual hygrobutanol method by Johansen (1940). Staining was carried out in a 1% aqueous safranin solution and the embryos were counter stained in a solution containing one part 0.5% fast green in 95% ethanol and one part methyl cellosolve for fifteen minutes.

#### (6) Measurement of Ovules

The dimensions of ovules, harvested at different stages of dev-

elopment, provide a measure of ovule growth. It is of interest to find if there is a pattern of growth and if it can be related to either seasonal or developmental changes.

The length of each ovule was measured from median sections, taken parallel to the upper surface of the ovule. Measurement was from the lower point of the micropylar canal to the outer integument cells at the chalazal end. The measurement was not taken from the outer end of the micropylar canal because the integument around it was often buried in the cone axis tissue and broke off during dissection.

Ovule width was measured between the edges of the outer integument on each side, at the point where the integument becomes free from the nucellus. The length of the megaspore was measured from its median section.

An eye piece micrometer was used at a magnification of 160 x and the divisions were calibrated against a stage micrometer. The units of measurement were equivalent to 0.039 mm.

The accuracy of measurement was affected by several factors. Often the ovules were cut obliquely and in obvious cases such ovules were not measured. When the ovules became longer than the 0.39 mm scale they had to be moved so that a complete measurement could be made. On the other hand, when the ovules were small, just after pollination, the differences between daily harvests were small and were masked by variation between cones in stages of development, and by errors in measurement due to orientation.

In chapter IV it was shown that ovules in the lowest part of the normal ovule bearing zone appear to be smaller than the rest. The amount of pollen they could accommodate and measurement bore this out.

Therefore, a mean value for the ovule dimensions was obtained for each cone. However, the number of ovules from which the mean was derived varied between cones. Even when only two ovules in a cone were suitable for measurement, their mean dimensions were plotted. Since the graphs

(figures 5:2 and 5:3) show dimensions to only one decimal place, smaller variations, due to error, are no longer apparent.

### III. OBSERVATIONS AND RESULTS

#### (1) Pollen Germination

(a) Introduction. Although pollen germination is not strictly a feature of female development, it occurs within the female cone, at a particular stage of cone development and is essential to continued ovule growth. Because of its importance from the point of view of seed production success, observations on pollen germination will be reported here. Furthermore, some aspects of germination of pine pollen in vivo have not been covered in the literature.

(b) Timing of Germination. The time that pollen germinates after reaching the micropyle probably varies between species, but is generally between two days and a week (Ferguson, 1904; Thomas, 1951; Sarvas, 1962; Nekrasova, 1970). These authors do not mention that the time of germination could vary within species or within clones. In P. radiata pollen germination had begun in some grains when cone development was assessed at EC (early closure). About this time the micropylar arms of some ovules (seen in sections) had swollen, constricting the micropylar canal. In chapter IV (table 4:4) it was shown that, in unbagged cones, the period between the end of receptivity (first day cone was assessed at LR) and cone closure can last from nine to fourteen days. It was also shown that pollen can reach ovule micropyles in a five to ten day period before that. It is difficult to see whether pollen germination begins immediately on its arrival in the micropyle, since physiological changes may well be occurring, but the fact that pollen can get into micropyles during the receptive stage, while pollen tubes cannot be identified until early closure up to two weeks later, suggest that there is some delay between arrival in the micropyle and germination. If germination does occur about two weeks after pollen

arrival, the time taken for germination to occur is longer in P. radiata than in other species described in the literature.

Because pollen grains reach the micropyles of different ovules on different days (or nights), they would have been in an environment conducive to germination for a variable time, so it could be expected that, in one cone, and even within an ovule, some grains would germinate before others. This can be seen in P. radiata by looking at microscope slides of cones harvested at EC. It can also be shown by counting the proportion of viable grains which had germinated in cones harvested at intervals, following pollination. Such results are shown in figure 5:1. Cones of clone 89 from Whaka (1970) were pollinated at receptivity, although some would have been receptive longer than others. A self pollinated cone and a cone pollinated with a pollen mixture were harvested at daily intervals from the fifth day after pollination. Very little pollen had reached some cones, possibly as a result of the application technique or the stage of cone development at the time, so in these cones no results were obtained. In cones which had received more than ten grains in the number of ovules observed (this varied because whole cones were sectioned), there was no sign of germination until the period between the seventh and the ninth day after pollen application, when the exine of a few grains in some cones appeared to be thinner and bulged slightly on the ventral side. Because this was difficult to detect with certainty, it was not recorded as definite germination. After the tenth day a small proportion of pollen grains had pushed out definite lobes. (Inviabile pollen grains, which looked misshapen, contained dense cell contents and a bright red, misshapen generative cell nucleus, were not counted.) From the tenth to the fifteenth day after pollination the proportion of germinated grains in each cone increased. All the viable pollen in the two cones harvested after thirty days had germinated. Anomalous counts came from three cones, harvested on the fifteenth and sixteen days, but these cones might have been at a slightly earlier develop-



mental stage so that the pollen could have been moved to the micropyles later. Germination proportions in self pollinated and out pollinated cones are comparable and give no evidence for suspecting that a partial barrier to self pollination could exist at this stage.

In figure 5:1 germination proportions in cones from clone 121, which were not artificially pollinated and which were debagged two to three weeks after being assessed at receptivity, are also shown. The proportions are plotted against the number of days after debagging, and therefore after pollen arrival. In this clone, also, there was an increase in the proportion germinated over a period of more than a week. But, the germination begins after a shorter period, following pollen arrival (six days). Cone development, which was probably more advanced than is usual at the time of pollen arrival, could have influenced pollen germination in some way, but more certain information cannot be determined from a morphological study. Alternatively, germination commencement could have been enhanced by increased environmental temperatures or clonal differences in female tissues.

The closure of the micropyle by the enlargement of cells of the inner integument is comparable to that described for other species (Ferguson, 1904; Thomas, 1951), and can be seen by comparing photograph 16D with photographs 24G and 26A.

Pollen germination and closure of the micropyle occur at much the same time, but it would be expected that, in order to provide a closed environment for germination, micropylar closure would precede pollen germination. This was not always so. Just as pollen grains in one cone do not germinate simultaneously, micropylar closure is not simultaneous either. While the inner cells of the micropylar arms of some ovules had swollen, others, <sup>in</sup> pollinated and unpollinated ovules had not. In later harvests, however, all micropyles were closed.

The fact that micropylar arms appear to be either swollen, completely closing the micropyle, or not swollen at all, suggests that the

process of closing occurs very rapidly, or occurs at night. But, the dehydration and fixation of ovules could affect cells in the process of swelling so that they are not apparent in slides.

(c) Structure of the Pollen Grain at Germination

When pollen arrives in the micropyle it contains two degenerating<sup>t</sup> prothallial cells, a generative cell and a tube nucleus. (Ferguson, 1904). Although Ferguson used the term generative cell in a different sense, it will be used here instead of her term antheridial cell, in accordance with more recent useage (Thomas, 1951; Konar, 1960; Sporne, 1965; Stanley, 1967). The generative cell and tube nucleus can be seen in a P.radiata pollen grain in photographs 24F to H. The pollen wall on the ventral side of the grain is thinner and the pollen tubes arise at this point. (Thomas (1951) showed this in P.virginiana also.) The difference in thickness of the pollen wall can be seen in photograph 24E and the region of pollen tube emergence in photographs 24A to H.

(d) Growth of the Pollen Tube. Variation in the orientation of pollen grains against the nucellus tip was discussed in chapter III. Thomas (1951) noted that pollen tubes can curve around the grain in order to penetrate the nucellus. This also occurs in P.radiata and the pollen tubes can penetrate the nucellus, irrespective of the grain's orientation (photograph 29A, illustrating the spermatogenous nucleus, shows this). Occasionally P.radiata and P.virginiana (Thomas, 1951) grains trapped in the micropylar canal can germinate there and grow down to penetrate the nucellus. Very rarely such grains penetrate the tissue of the micropylar arms (photographs 45A and B). But, as Thomas (1951) noted, pollen grains almost always germinate in the micropyle only, even though conditions in the rest of the cone are extremely moist. Nevertheless, pollen incubated in vitro at 25°C on a drop of water, will germinate within twentyfour hours and grow. That it does not do so in the cone suggests that the surrounding tissues of the cone produce substances which are inhibitory to pollen germination and possibly fungal

growth. The in vitro pollen culture rapidly became infected with fungi but, fungal growth was never observed in the micropyle or inner cone surfaces.

In vitro germination often results in two directional growth of the pollen tube (Ho and Sziklai, 1970). Photographs 24B to D of P. radiata pollen show that two pollen tubes had begun to emerge from each grain, but, after thirty six hours of growth, one tube had become dominant. In a thousand pollen grains observed in vivo only one grain was seen with two points of origin. There is evidence then, that, in natural conditions, pollen germination and the direction of tube growth are affected by endogenous factors. Hormones, which affect pollen germination and growth, have been extracted from young cones by a number of workers (Hashizume and Kondo, 1962; Stanley, 1967).

Pollen grains reaching the micropyle are well supplied with starch grains (Ferguson, 1904). In P. radiata their distribution in the pollen grain and tube, which has been germinated artificially in water and stained with iodine solution, is variable (photographs 24A to D).

When pollen germinates and penetrates the nucellus, the tube nucleus moves into the pollen tube (Ferguson, 1904; Konar, 1962). The tube nucleus of P. radiata pollen is shown in photographs 24F, G and H. Pollen tube growth is, at first, rapid, as described by Thomas (1951) for P. virginiana also, and after the tubes have penetrated several cell layers, they may branch. About six weeks after pollination in P. radiata (towards November), the tubes cease growth towards the megaspore mother cell, and during the summer and winter, slowly ramify the nucellus cap region, with subsidiary tubes of varying diameter. (photographs 27A, D and E). During the winter the main pollen tube enlarges.

## (2) Growth of the Ovule

The size of ovules at each stage, and the pattern of ovule growth can be seen in figures 5:2 and 5:3, in which mean ovule length and breadth, and megaspore length are plotted for each cone sampled.

Just after pollination the ovule is about 0.3 mm in length and in ten days increases by another 0.1 mm. In the following six weeks (September to mid October) the rate of growth is slower (0.2 mm in six weeks). From the end of October (about the time of meiosis) to the beginning of January (after the first division of the megaspore nucleus) the ovule increases from 0.6 mm to 1.3 mm, then ceases growth. Growth is resumed at the end of June (winter) when the megaspore has more than a hundred nuclei. It continues at a greater rate than at any previous stage until mid September, almost a month after fertilization. The length reached at this stage averaged 5.7 mm, but could vary within and between clones.

The pattern of ovule growth follows the pattern of cone growth shown by Sweet and Bollmann (1971). It agrees with Ferguson's statement (1904) that ovule growth in pines is slow after pollination in early summer, but becomes rapid in the following spring. However, neither she nor any other author shows the growth pattern of ovules by the changes in their dimensions. Resting periods are deduced from the behaviour of the megaspore nuclei. Data from the literature, summarized in table 5:2, shows that female meiosis in pines usually occurs in the spring or early summer, and the first division of the megaspore nucleus usually takes place from four to six weeks later. After several divisions a resting period is reached (beginning in summer in P. roxburghii, P. virginiana, P. wallichiana and P. gerardiana and at the end of autumn in P. austriaca, P. rigida and P. strobus). Nuclear divisions are resumed at the end of winter, or in spring.

Clones 19 and 55 of P. radiata at Kaingaroa undergo meiosis in spring and the first division of the megaspore occurs eight to ten weeks later. P. radiata differs from these other species in that nuclear divisions in the megaspore occur from late summer and throughout winter, even though the ovule does not increase in size. Eight or nine divisions occur prior to the period of rapid spring growth. The accompanying morphological development will be described in the next section.

Species & author	(1) Devel. Stage	(2) Time of Stage	(3) Season	(4) Time after Previous Stage (from column 1)
<u>P. rigida</u>	meiosis	mid June	early summer	
<u>P. austriaca</u>	1st div.	end July		6 weeks
<u>P. strobilus</u>	2nd div.	1st week Aug.	summer	4-5 days
Ferguson (1904)	5th div.	mid October	autumn	10 weeks
	rest	till April	mid spring	26 weeks
<u>P. roxburghii</u>	meiosis	beginning Mar.	spring	
Sethi, (1928)	1st div.			no record
	2nd div.	May	late spring	about 8 weeks
	rest	Till Feb.	late winter	36 weeks
<u>P. virginiana</u>	meiosis	May	late spring	
Thomas (1951)	1st div.	October	mid autumn	20 weeks
	2nd div.	October		'rapidly'
	3rd div.	January	mid winter	12 weeks
	continued divs.	till May	late spring	16 weeks
<u>P. wallichiana</u>	meiosis	Apr. to mid may	late spring	
Konar (1960)	1st div.	June	early summer	4-6 weeks
	rest	till Feb.	late spring	33 weeks
<u>P. gerardiana</u>	meiosis	beginning June	early summer	
Konar (1962)	4th div.	beginning July	summer	4 weeks
	5th div.	end July	summer	3-4 weeks
	rest	till March	spring	28 weeks

Table 5:2 The timing of megaspore divisions in different species.

Clone	Year	Estimated R	Date when meiotic figures found.	Time from R
55	1968	14th Aug.	18th Oct. (2 cones)	9 weeks
19	1968	16th Aug.	18th Oct. to 6th Nov. (2 cones)	9- 11 weeks
7	1970	21st Aug.	6th to 20th Oct. (12 cones)	$6\frac{1}{2}$ - $8\frac{1}{2}$ weeks
121	1970	7th Aug.	29th Sept. to 6th Oct. (6 cones)	6- 7 weeks
372	1970	2nd Sept.	before 14th Oct. (12 cones)	up to 6 weeks <del>±</del>

Table 5:3      Dates when cones were found to contain some ovules with megaspore mother cells in the process of meiosis. Dates of estimated receptivity are given so the approximate interval between pollination and meiosis can be calculated.

\* Some cones were sprayed with extracts from pollen grains, but meiotic divisions in them were as frequent as they were in artificially pollinated cones of the same clone.

(3) Post Pollination Development, Meiosis and Subsequent Megaspore Growth

(a) Growth of the Megaspore Mother Cell and Differentiation of the Nucellus, Spongy Tissue and Integument up to the Time of Meiosis.      The degree of growth and differentiation in

the first two months after pollination can be seen by comparing photographs 25E and 26A with photograph 17C. The changes are similar to those described for other species, but in other species meiosis occurs only a week or two after pollination, whereas in P. radiata it occurs from six to ten weeks later (table 5:3).

During the two months following pollination the nucellus cap cells enlarge, develop thicker cell walls and accumulate numerous starch grains. The nucleus of the megaspore mother cell enlarges and stains more readily than at pollination, but the end walls of the cell remain pointed (photographs 17C and 25A). Sethi (1928) found that the megaspore

mother cell of P. roxburghii became conical, being broader at the chalazal end. In P. radiata, as in other species of Pinus, spongy cells, orientated around the megaspore mother cell, proliferate and develop dense cytoplasm, which gives the appearance of a closely packed tissue about five cells deep. The outer layer is often flattened against the nucellus cells (photographs 25A to D). Where the nucellus and integument cells are in contact occasional integument cells become elongated and produce densely stained contents. At pollination the integument is a uniform band of cells but it gradually develops a middle zone of smaller, round cells, which stain brightly because their nuclei are close together.

(b) The 'Connecting' Tissue. At the deepest point of the ovule there is a break in the middle integument zone, filled by parenchyma cells, orientated at right angles to it. These cells are continued in the ovuliferous scale tissue and form a band of small, thin walled cells with dense cytoplasm, passing between the larger, often densely stained, scale cells. The parenchyma band can be traced to one of the vascular bundles of the ovuliferous scale, running below the ovule (photographs 25E and 27A).

Because the ovule is a growing organ which remains dependent on the parent tree for fifteen months after pollination, it must absorb nutrients from the tree. Since it is not in direct contact with the vascular tissue of the ovuliferous scale it is possible that this band of cells provides a 'path of least resistance' for the enhanced transport of nutrients from the vascular cylinder to the ovule.

Although the parenchyma 'connecting' band is easily seen in vertical longitudinal sections of the cone scale, it has not been described in the literature. Ovules are often removed from the ovuliferous scale, or are sectioned in the horizontal plane, either of which possibility may account for the absence of comment on a tissue which must have an important function. The development of this tissue will be described in later sections of this chapter.

(c) Meiosis. Meiosis usually occurs in the megaspore mother cell of P.radiata in October (late spring) at Kaingaroa (table 5:3). The timing of meiosis varies between cones. It possibly varies also between clones, years and sites. Meiotic figures were found in cones of one clone over about two weeks, a similar period to that found in P.virginiana by Thomas (1951). However, within a P.radiata cone, megaspore mother cells are found in varying stages of meiosis.

The main stages of meiosis can be seen in photographs 25A to E, and are fully described by Ferguson (1904). Two cells, the diad, are formed from the first division, but only the cell at the chalazal end of the diad divides for a second time, giving rise to a triad of megaspores. Only the cell at the chalazal end of the triad continues to grow. The others degenerate and disappear after a few weeks. In P.austriaca (Ferguson, 1904), P.wallichiana, P.roxburghii and P.larico (Konar and Ramchandani, 1958) a tetrad of megaspores is formed and Ferguson (1904) notes that in some species both triads and tetrads occur. Thomas (1951) found one tetrad and many triads in P.virginiana, but no tetrads were seen in the P.radiata clones observed.

(d) Growth of the Functional Megaspore. The functional megaspore enlarges quickly, becoming vacuolate, and the cytoplasm with the nucleus attached, moves to the periphery of the cell. In most cases the central vacuole collapses during the fixation process (with F.A.A. or with acrolein and gluteraldehyde). The cytoplasm is then seen as a configuration in the centre of the cell as in photograph 28A.

By January (two months after meiosis) the ovules of P.radiata have ceased to increase in size, but the megaspore continues to enlarge and at the end of December, or in January (summer), the megaspore nucleus divides. This occurs at different times in different cones, and even in different ovules within a cone. The approximate timing of this division, relative to meiosis, is shown for other species in table 5:2.



Because the megaspore contains haploid nuclei it may also be called the female gametophyte, but, for simplicity, the term megaspore will be retained for the multinucleate structure.

Linear growth of P. radiata megaspores is shown in figure 5:3. From this it can be calculated that the megaspore increases in volume eight times between March and May, while the number of nuclei increases from eight to thirty two. From May to July it increases six times in volume, by which time two hundred and fifty six nuclei are present. The number of nuclei from July onward is impossible to count in serial sections. By September they are crammed along the megaspore wall and at this stage cell wall formation, which will be described in the next section, occurs.

(e) Development of the Spongy Tissue. About the time of meiosis in P. radiata, as in other pines (Ferguson, 1904; Thomas, 1951; Singh and Johri, 1972), the spongy cell nuclei increase in size. In photographs 25A, C, D, and E and 26D the size of the spongy cell nuclei can be compared with the size of the nuclei in the nucellus cells. In P. radiata the spongy cells enlarge and become vacuolate about the time of the first division of the megaspore nucleus (photograph 26C), but Ferguson (1904) does not describe this stage for P. strobus. Cell divisions in the spongy tissue allow it to keep up with the enlarging megaspore until July, but because of this increase in volume in the centre of the ovule, the inner nucellus cells in P. strobus (Ferguson, 1904), P. virginiana (Thomas, 1951) and P. radiata become flattened and even degenerate (photographs 26C, 27A and D). The effect is increasingly noticeable in P. radiata from May onward, even though the ovule recommences growth in June.

By July the spongy tissue is stretched into two or three layers surrounding the megaspore. Many of the cells appear to be attached to a common base, lining the nucellus cells. Ferguson (1904) considers that this base is made up of flattened, degenerated inner nucellus cells

the contents of which are being absorbed by the spongy cells. Since flattened, often degenerating inner nucellus cells persist in P. radiata ovules from May to mid October and since, at the end of this period, the number of normal cell layers is comparable to the number present at the beginning, it seems likely that the inner nucellus cells are being absorbed and replaced by cell divisions in the outer layers. This may provide nutrition and space for the enlarging megaspore. From June to September the cytoplasm of the spongy cells increases greatly in density and appears to condense around the nuclei. The cells become detached from one another (photographs 28A, B, E, and G), but the outer cells remain attached to a rather amorphous, multilayered base (photograph 28B). From August on some spongy cell nuclei are seen to be in various stages of mitotic division, and often two nuclei can be seen in one clump of cytoplasm (photograph 28B). By mid September in some ovules, and October in others, the spongy cell nuclei and cytoplasm degenerate and stain deeply, having apparently fulfilled their function (photograph 31).

(f) Changes in the Pollen Tube. Towards the end of autumn, or in early winter, the generative cell of the pollen grain divides, giving rise to the sterile (= stalk) cell and the spermatogenous (= body) cell. Although stalk and body cell have been commonly used terms (Chamberlain, 1935; Thomas, 1951; Konar, 1958; McWilliam, <sup>and Mergen</sup> 1958), the terms given by Sporne (1965) will be used here because they express most clearly, the function of these cells.

The time that the division of the generative cell occurs varies between pollen grains, even within one ovule. The most advanced pollen grain had formed sterile and spermatogenous cells on the 24th April, 1971 (photograph 27A). Dividing generative cells were seen in ovules from 2nd May, 1969 in clone 55 and 2nd May, 1971 in clone 19 (photograph 27B). In other grains, harvested at the end of June, the generative cell was still visible. Ferguson (1904) also noted great varia-

tion in the timing of this division, within species as well as between them.

At first the sterile and spermatogenous cells are of equal size and lie side by side. Gradually their cytoplasm increases and their cell walls are no longer visible. The spermatogenous nucleus enlarges greatly and in P. radiata can sometimes be distinguished from the sterile cell by the end of June (photograph 27C). It is seen in most P. radiata pollen grains by mid August. Photograph 27E shows a pollen grain in which the spermatogenous nucleus is only a little larger than the sterile nucleus, and the tube nucleus, with a bright red staining nucleolus, lies toward the bottom of the tube. In P. virginiana<sup>r</sup> (Thomas, 1951) the tube nucleus also has only one nucleolus.

During September the enlarged spermatogenous nucleus and its cytoplasm moves into the pollen tube, followed by the sterile cell. A spermatogenous cell at this stage is shown in photograph 29A. The two cells come to lie above the tube nucleus in a clump of cytoplasm attached by cytoplasmic strands to the cell wall. In P. radiata the spermatogenous cell nucleus can be distinguished from the tube nucleus because it has four nucleoli rather than one. The sterile cell in P. radiata does not stain readily and is usually difficult to see from this time on.

Thomas (1951) noted that the time of migration of the spermatogenous cell of P. virginiana varied by a month in different pollen grains. This appears to be true of P. radiata also. However, little starch was reported in the pollen tubes of P. virginiana at this time, although there is plenty in those of P. radiata.

#### (4) Ovule Development from Prothallus Formation to Maturation of the Egg Cell

(a) Differentiation of the Nucellus and Integument. By the time cell wall formation occurs, the ovule has elongated considerably. Figure 5:3 shows that the increase in ovule width (once growth recommences in June) no longer follows the rate of increase in length. Frequent divis-

ions in the cells below the nucellus cap and greater longitudinal elongation of the megaspore account for the change in ovule shape (compare photographs 27D and 28A). By the end of winter the nucellus cap cells around the pollen tubes are almost all degenerated and are filled with deeply staining material. Outside the degenerated cells the nucellus cap cells are thick walled with sparse cytoplasm and the lowermost cap cells, below the pollen tubes, are almost full of starch grains.

Inner integument cells of ovules harvested in September are large and elongated with pointed end walls. They are separated from the nucellus cells by a single line of cells containing deeply staining material, but these cells are discontinued at the chalazal end, opposite the point where the 'connecting' cells pass out into the ovuliferous scale (photograph 28G). From September to November the outer walls of the outermost middle integument cells become thickened and contain granules of crystalline appearance. These form the line between the ovule and scale where abscission occurs later (photographs 30D and 31). A narrow zone of outer integument cells separates the scale cells from the middle integument (= stony layer, photograph 28G).

(b) The 'Connecting' Cells. Photograph 28G is a vertical longitudinal section, which shows that the ovule is separated from the vascular tissue of the scale by only two or three rows of scale cells. Because the band of 'connecting' cells curved away from the plane of sectioning, it was not possible to show its relationship to the vascular tissue of the ovuliferous scale in this photograph.

By September the 'connecting' cells are seen to be differentiating from parenchymatous cells in the inner integument tissue. They are thicker walled in the chalazal region and orientated at an angle to the lateral inner integument cells. At the chalazal point they are elongated at right angles to the middle integument cells, and pass through them into the scale tissue. The cells passing through the integument often have misshapen, degenerating nuclei and are extremely long with

very thick walls, features which suggest that they are differentiating as conducting cells (photograph 30F). Electron microscope studies might show developmental parallels between these cells and the cells of the vascular cylinder. The differentiation of this tissue advances into the ovuliferous scale from September to mid December. In photographs 30A to C it can be seen that the 'connecting' tissue has differentiated well into the scale by the beginning of November. The direction of differentiation is toward the vascular cylinder of the scale and the distance between the end of differentiation and the cylinder is connected by small cells (photographs 30B and C show them in transverse section). These cells differ from other scale cells in that they are smaller and rounder. They are never filled with deep staining substances. In later harvests the ovule was dissected from the scale so the progress of differentiation of the thick walled 'connecting' cells toward the vascular tissue was not seen. It seems likely that the differentiating cells would connect with the vascular cylinder of the scale, either before, or about the time that the middle integument layer became fully differentiated. This layer forms a protective seed coat which is composed of extremely thick walled cells. In P. radiata as in P. wallichiana (Konar and Ramchandani, 1958) the thick walled cells appear before the embryos are fully formed (photographs 32G and H), when the seed probably requires nutrients from the rest of the plant. The 'connecting' cells could have differentiated to provide a nutrient conducting path through the impervious stony layer of the integument.

(c) Cell Wall Formation in the Megaspore. Cell wall formation around the numerous nuclei in the megaspore is usually difficult to detect because the fixation technique causes the megaspore cytoplasm to shrink. The cell walls are, at first, delicate and do not show up in the shrivelled tissue. Furthermore, cell wall formation occurs at different times in the ovules of one cone. In clones 19 and 55 from Kaingaroa, 1970 and 1972, cell wall formation took place around the middle of September,

just over a year after pollination. Megaspores in P. radiata at this time averaged 1.3 mm in length and 0.7 mm in width. However, in a single cone, and even on the same ovuliferous scale, ovules with either a megaspore with peripheral cytoplasm, or a cellular prothallus may be found. Cell wall formation was seen in a few ovules and appeared similar to that described by Maheshwari and Singh (1966) for the development of cell walls in the female gametophyte of gymnosperms. 'At the end of the last mitosis secondary spindles develop so that every nucleus becomes connected by spindle fibres to six adjacent nuclei'. These were seen in a few P. radiata ovules (photographs 28C and D). Anticlinal walls are laid down centripetally, forming cavities called alveoli, which are open at their inner ends. The nuclei lie at the open ends and the persisting spindles seem to guide the laying down of the wall material (photograph 28D). According to Maheshwari and Singh (1966), the closure of an alveolus is initiated by the lagging of its nucleus behind wall formation. This is seen in P. radiata in photograph 28E.

Because this stage was found so rarely, it seems that the nuclei of the alveoli must divide soon after the cells are closed. The large number of nuclei, the very delicate cell walls and an increase in cytoplasm result in a dense prothallus, which shrinks in F.A.A. (photographs 28G and 29B). With time the prothallial cell walls become thicker, the cells enlarge and the dense appearance is lost. (photograph 29C).

(d) Archegonial Development. According to Ferguson (1904) and Thomas (1951), archegonial initials usually differentiate after cell wall formation in the megaspore, but can occasionally be seen before it is complete. The number of archegonia formed in different species is given in table 5:1. Variation in the number found in P. radiata will be discussed in the following chapter. In photograph 28E five cells at the micropylar end of the gametophyte are narrower than the other cells and have slightly larger nuclei, supported by numerous cytoplasmic strands. They occur in the position where archegonia can be seen at the end of

September. Because most of the prothalli were shrivelled, the archegonial initials usually could not be identified, but they probably develop according to the description given by Ferguson (1904). Photograph 28F shows an archegonial initial in P. radiata.

Soon after this stage the archegonial initial nucleus divides, giving rise to a primary neck cell nucleus and the central cell nucleus. The primary neck cell divides by an anticlinal wall, giving two neck cells. These divide again, forming a tier of four cells, two of which can be seen side by side in sections (photographs 29B and D). A later division of the neck cells can result in a second tier, so the number of neck cells varies between ovules (Blackman, 1898; Ferguson, 1904; Thomas, 1951). The left hand ovule in photograph 29C has two tiers of neck cells, while the right ovule has one. It is not uncommon for only one or two of the first tier cells to divide again, so forming a one or two celled second tier, and four, five, six or eight neck cells in all.

By the end of September in some ovules, and by the first few days of October in others, the archegonia of P. radiata can be seen as enlarged, clear central cells, each beneath the first neck cells on the micropylar edge of the prothallus. Sometimes jacket cells (= sheath cells, Ferguson, 1904) can be seen along the central cell border, because their cytoplasm is denser than the cytoplasm of other prothallial cells (photograph 28F). From mid October to November (photographs 29B to E) the archegonia enlarge. The central cell cytoplasm develops many vacuoles. This has been referred to as the 'foam' stage by Singh and Johri (1972). The jacket cells have continued to divide to keep up with the growth of the central cell and their nuclei enlarge, but the cells themselves have not grown as much as the remaining prothallus cells. These prothallus cells (in P. radiata as in P. virginiana (Thomas, 1951) and P. strobus (Ferguson, 1904)) divide and enlarge, so that the prothallus forms lobes beyond the archegonium, leaving a depression above the neck cells (photographs 29C and 31). The jacket cell walls against the central cell are covered in large

pits, and the wall, in tangential section, looks like a reticulate network. In well fixed sections thickened regions of the wall can be seen in cross section (photographs 29E and 33D). P.A.S. stains these thick regions bright purple. The thickened walls of the central cell have also been described by Mathews (1932), Thomas (1951) and Konar (1962) for three other species of Pinus.

In the second week of November, 1970, at Christchurch and Kaingaroa and November, 1971 at Whaka, the vacuoles in the central cell cytoplasm had disappeared. In P.nigra (Camefort 1965 ) the central cells, at this stage, contain islets of cytoplasm which are isolated by a double membrane derived from endoplasmic reticulum. Similar bodies seen in the cytoplasm of other species were previously thought to be proteid, or food bodies (Ferguson, 1904; Chamberlain, 1935; Thomas, 1951). They can be seen in the cytoplasm of the central cell in P.radiata (photograph 29F) and in the egg cells (photographs 33D, 34A and B).

By the second week of November the lengths of seven archegonia from Christchurch ovules ranged from 0.65 mm to 0.92 mm with a mean of 0.73 mm (measurements from the inside of the jacket cells). At the same time the lengths of <sup>u</sup>fourteen archegonia from Kaingaroa ranged from 0.64 mm to 0.77 mm with a mean length of 0.71 mm. Variation in archegonial length within cones is caused to some extent by the arrangement of archegonia within the prothallus. When there are two or more archegonia, side by side (in sections through the broad plane of the ovule), they are longer and narrower than a single archegonium in front, or behind, its neighbours. (Compare the shape of the archegonium in photograph 33D with those in photographs 34A and B).

The arrangement of archegonia also affects the amount of prothallial tissue between them at their point of greatest contact. When there are only two archegonia in the prothallus there is usually a row of prothallial cells between the two jacket layers at the point where the archegonia are closest together (photograph 34A). But, sometimes the jacket



cells may contact one another at the closest<sup>t</sup> point (photograph 34B). When there are three or four archegonia the jacket cells appear to be crushed and at the middle point may merge as a mutual jacket (photograph 35E). This has also been described for P. virginiana (Thomas, 1951).

Very shortly before fertilization, when the pollen tubes have recommenced growth, the central cell nucleus of P. radiata divides (photograph 29F) in the manner described for other species (Blackman, 1898; Chamberlain, 1899; Ferguson, 1904; Thomas, 1951). This gives rise to a ventral canal cell and an egg nucleus. The ventral canal cell nucleus degenerates as soon as it is formed, but a thin cell wall lies beneath the neck cells. The egg nucleus moves rapidly to the centre of the egg and enlarges greatly (photograph 29G). The time between the formation of the egg nucleus and fertilization is probably short. On the 16th November, 1970, ovules from several cones of clone 55 were mainly in stages of fertilization, but a few still contained central cell nuclei or were in the process of forming the ventral canal cell and the egg nucleus. In clone 19, ovules harvested on the 3rd November, 1970 contained central cells in the foaming stage or with dense cytoplasm and central cell nuclei in the prophase condition. However, within ovules of P. radiata and P. roxburghii (Sethi, 1928), the archegonia were all at the same stage of development. Ferguson (1904) considers that the time from the division of the central cell nucleus to fertilization is about five days in Pinus, but implies that it may be shorter in species where the pollen tube has reached the prothallus at the time the central cell divides. P. radiata pollen tubes have usually reached half the distance to the archegonia at this time. McWilliam<sup>and Mergen</sup> (1958) found fertilization stages within one P. nigra tree over a five day period, but he did not mention the formation of the egg nucleus. It would require daily harvesting of a large number of cones to determine the exact time over which fertilization occurs.

(e) The Megaspore Membrane. From cell wall formation onward the

megaspore cell wall of gymnosperms becomes thickened (photograph 28E), and is termed the megaspore membrane (Thomson, 1905). The megaspore membrane surrounds the prothallus and is thicker at the chalazal end. From the time the archegonial initials are formed, it stains differently from the cell walls of other tissues. Pettitt (1966) reported that it is sudanophilic (i.e. classified broadly as lipid). Thomson (1905) found that the inner third of the megaspore membrane stained with haematoxylin, while the rest stained with safranin, but also showed that there were two layers to the megaspore membrane by staining with chlor-zinc iodine. As long as the section through the membrane is not oblique, with careful focusing under high power, it can be seen that in P.radiata the inner edge is stained more densely (photograph 32A). The inner surface of the megaspore membrane, in P.radiata, as in other species, is smooth, while the outer surface is piloid (photographs 32A and D). A diagram, after von Lürzer (1956) is given by Maheshwari and Singh (1967) for the megaspore membrane of pines, and is very similar to that of P.radiata.

By the time the ventral canal cell is formed the micropylar region of the megaspore membrane is very thin, or stretched, and soon afterwards the prothallus appears to grow beyond it (photograph 32D). This means that the pollen tubes do not have to penetrate the megaspore membrane in order to fertilize the egg. According to Thomas (1951), the megaspore membrane is a thick walled structure composed of flattened cells of the spongy layer, closely adhered to the prothallus. Pettitt (1966) also considers that the megaspore membrane represents the remains of the spongy tissue in gymnosperms. However, in P.radiata the spongy tissue persists in a degenerated form for some time after the megaspore membrane is evident and is, itself, attached to a compressed layer of inner nucellus cells (photographs 28B and E). It is possible that the fixation technique used by Thomas allowed the spongy tissue to remain closely attached to the megaspore membrane. The fixation of P.radiata ovules for this study caused the prothallus and megaspore membrane to shrink away from the

surrounding tissues so that the megaspore membrane surface could be clearly seen in some sections. There was no evidence of nuclei attached to the megaspore membrane, or of cell walls within it (photograph 32B). However, the swollen tips of the pilli gave the effect of areas of greater density, in the surface view and could have lead to the belief that the megaspore membrane is derived from the spongy cell remains.

The view held by Thomson (1905) that the megaspore membrane is homologous to the wall of the pollen grain and is of similar composition is more generally held (Maheshwari and Singh, 1967; Singh and Johri, 1972). There is no suggestion in the literature of the function of the megaspore membrane. From the pilloid nature of the outer layer and the food storing qualities of the prothallial cells, it might be suggested as having a nutrient absorbing function. A thorough developmental study with an electron microscope might clarify both the homologies and the function of the megaspore membrane.

(f) Division of the Spermatogenous Cell. According to Dixon (1894) the spermatogenous nucleus in P.sylvestris divides to give two sperm nuclei, shortly after the generative cell has divided. Ferguson (1904) found, for the species she studied, that the spermatogenous cell did not divide until it had descended into the pollen tube. This was true also for P.radiata. In some clone 19 ovules harvested on the 3rd November, 1970, and in clone 55 on the 6th November, 1968, the spermatogenous nucleus was enlarged and contained dense chromatin (photograph 32E). In a few tubes, two sperm nuclei were seen. These were not as large as the two sperm nuclei seen in harvests from the 16th November, 1970. Compare photographs 32F and 33A. Thomas (1951) showed that, in P.virginiana, the formation of the sperm nuclei preceded the division of the central cell nucleus by several days, but in P.wallichiana (Konar and Ramchandani, 1958), the spermatogenous nucleus often divided when the megaspore was in the free nuclear stage, although the time of this division varied. In P.radiata ovules, harvested on the 3rd November, 1970,

the central cell nucleus had not divided, but in ovules with two sperm nuclei, the central cell nucleus appeared to be in the prophase stage, and the cytoplasm was denser than in those ovules still containing pollen tubes with spermatogenous nuclei. But, although central cell divisions were simultaneous within an ovule, the divisions of the spermatogenous nuclei within an ovule did not appear to be simultaneous.

#### (5) Seed Development

(a) Timing of Fertilization. Fertilization is the process by which the haploid male (sperm) nucleus is carried to the haploid female (egg) nucleus, and the two are combined in one cell, the zygote. Once the zygote is formed the ovule becomes a seed. Not all the steps of fertilization described in the literature (Blackman, 1898; Ferguson, 1904; Chamberlain, 1899; Haupt, 1941; McWilliam, <sup>and Mergen</sup> 1958) were found in the P. radiata cones harvested, but the observed stages were typical of those described for other species of Pinus and will be described below.

In clones 19 and 55 from Kaingaroa, harvested in mid November, 1970, different stages of fertilization were found. In some ovules the egg cell had not been formed (photograph 31), but in others the sperm nucleus was in contact with the egg nucleus (photograph 33D). In mid November, 1972, ovules from Whaka had been fertilized. In ovules from one cone, they were seen in stages from four cell proembryo formation to sixteen cell proembryo extension- comparable development to ovules harvested at the end of November, 1970, at Kaingaroa and in mid December, 1968, also at Kaingaroa. In contrast to this variation, uniformity in development at fertilization and proembryo formation within one cone was seen in P. larico (Chamberlain, 1899) and P. monophylla (Haupt, 1941), but P. lambertiana (Haupt, 1941) showed similar variation to that of P. radiata.

(b) Growth of the Pollen Tube. While the egg cell was forming and moving to the centre of the egg, one tube from each pollen grain was growing through the nucellus tissue. In P. nigra (McWilliam, <sup>and Mergen</sup> 1958) this phase of pollen tube growth lasted ten days. In P. radiata pollen tubes

do not usually grow directly towards the depression above an archegonium, but once they reach the fissure between the nucellus and prothallus, they turn and grow along it to an archegonial neck (photographs 33B and 35A). When there is more than one, the leading tubes grow well apart, even though the overwintering tubes may, in some regions, be in direct contact with one another. Because they grow well apart each tube nearly always succeeds in fertilizing a separate egg. When there are more tubes than archegonia, which is not uncommon (data is given in table 6:10), the unsuccessful tube may continue to grow for some distance around the side of the prothallus, or may begin to penetrate the prothallus. In other cases it ceased growth by the archegonial depression (photograph 45C). Unsuccessful pollen nuclei remain in the tube with the cytoplasm and degenerate.

(c) Fertilization. Successful pollen tubes growing down the depression above the neck cells part, or rupture, them (photographs 33C and 34B). Just before this the egg cytoplasm develops a large vacuole, the receptive vacuole, which lies just below the neck cells and the ventral canal cell (photographs 34A and B). This is typical of Pinus (Blackman, 1898; Ferguson, 1904; Sethi, 1928; Maheshwari and Sanwal, 1963). In P. radiata, as in P. roxburghii (Sethi, 1928), the receptive vacuole appears to form from the coalescence of several smaller vacuoles.

The contents of the pollen tube pass into the receptive vacuole, the egg cytoplasm or both. In doing so they must rupture the ventral canal cell wall, which remains intact in the unfertilized ovules (photographs 43C and D). Along with cytoplasm from the pollen tube, many starch grains pass into the egg, and, in older seeds stained with P.A.S., they can be seen deep in the egg cytoplasm. Starch grains passing into the egg have been reported in other species also (Dixon, 1894; Ferguson, 1904; Sethi, 1928; Konar and Ramchandani, 1958). In photograph 34A the smaller of the two sperm nuclei can be seen at the neck end of the egg. This appeared to be typical of the ovules observed. The tube and sterile

nuclei, however, were not identified in the P. radiata eggs, although they have been seen in other species (Blackman, 1898; Ferguson, 1904; Sethi, 1928; Konar and Ramchandani, 1958; McWilliam,<sup>and Mergen</sup> 1958; Camefort, 1969).

Photographs 33D and 34A and B show the sperm nucleus embedded in the egg nucleus. As Ferguson (1904) and Thomas (1951) pointed out for P. strobus and P. virginiana, respectively, there is no apparent distortion of the egg nucleus accompanying this. The sperm nucleus of P. radiata is about one third the length of the egg nucleus, similar to those in the species described by Blackman (1898) and Sethi (1928). Because a large number of ovules contained eggs at the stage where the two nuclei were in close contact, but retained intact nuclear membranes, it is considered that this phase probably lasts longer than other phases of fertilization in P. radiata. Although a few ovules had not been fertilized by the 16th November, 1970, the larger sperm nucleus, in those which were fertilized, was always deeply embedded in the egg nucleus, even though some cytoplasm was still passing between the neck cells. None were seen in the area between the receptive vacuole and the egg. Chromosomes of the first division of the zygote were seen in only one ovule from this harvest ( photograph 35B). By this time the nuclear membranes of both male and female nuclei have disappeared, and, as in other species of Pinus, the zygote spindle lies obliquely to the long axis of the egg (Thomas, 1951; Konar, 1958; Roy Chowdhury, 1962). In other species of Pinus, fusion of the two sets of chromosomes takes place on a multipolar spindle, which becomes bipolar (Ferguson, 1904; McWilliam,<sup>and Mergen</sup> 1958; Dogra, 1967). Photograph 35B shows a bipolar spindle.

Thomas reported that fertilization in P. virginiana is a prolonged process, but that the zygote divides immediately to give two small pronuclei, which enlarge and rapidly divide again. This is taken to be the first stage of proembryo development (Dogra, 1967). In P. nigra (<sup>and Mergen</sup> McWilliam, 1958) the first and second divisions of the zygote

are also rapid. The two cell stage of P.radiata was not seen. The earliest stage, following the first division of the zygote, was found in clone 55, harvested on the 12th December, 1968 (photograph 35C) and the 30th November, 1970, in which four free nuclei were situated at different points in the egg (photograph 35D).

(d) The Free Nucleate Proembryo. The four segmentation nuclei (Ferguson, 1904; Sethi, 1928) of P.radiata and other pines move towards the base of the egg and lie side by side in one plane (photograph 35E). Berlyn and Passof (1965) showed that fibres which are continuous with the thick nuclear membranes of the segmentation nuclei are also attached to the egg wall. They suggest that the fibres originate in the spindle and may be microfibrils associated with cell wall formation. They do not comment on the possibility that they are associated with the movement of nuclei to the base of the egg. Such fibres were not seen in these preparations of P.radiata seeds, possibly because the fixation process was not good enough.

Photograph 35E shows that the four segmentation (free) nuclei of P.radiata are surrounded by dense cytoplasm, which is distinct from the egg cytoplasm even though it is not separated from it by cell membranes. The appearance and staining ability of this cytoplasm has been described in other species by Ferguson (1904), Sethi (1928) and Thomas (1951). Camefort (1969) called this neocytoplasm. He showed that in P.nigra this is derived from the nucleoplasm of the zygote, but it receives some mitochondria from the perinuclear zone of the egg cytoplasm. The nucleoplasm moves to the base of the egg along with the four proembryo nuclei. In Larix decidua plastids from the male cytoplasm pass into the nucleoplasm at the base of the egg, but Camefort did not show this for pines. However, he was certain that the egg did not supply plastids to the neocytoplasm.

(e) Development of the Proembryo. The interval between the arrival of the four segmentation nuclei at the base of the egg

and their simultaneous division could not be determined for P. radiata Thomas (1951) concluded that proembryo development was rapid, because sixteen celled proembryos were seen five days after fertilization. In P. sylvestris (Sarvas, 1962) the suspensors had fully elongated and young embryos were developing eleven days after fertilization. In P. radiata many ovules from clone 55 were in stages of fertilization on the 16th November, 1971. On the 30th November, 1971, the majority were in the free nucleate stage. Proembryo development in P. radiata is therefore much slower than in P. virginiana and P. sylvestris.

The mitotic division of the four free nuclei of P. radiata is illustrated in photographs 35E, 36A and B. It results in eight nuclei, arranged in two tiers (i.e. two layers of cells lying in the horizontal plane). This structure is called the primary proembryo. The four upper nuclei are surrounded by egg cytoplasm, but the lower four remain in the neocytoplasm (photograph 36C). This has been described for P. virginiana (Thomas, 1951) and illustrated for P. larico (Chamberlain, 1935). Delicate cell walls form around the lower nuclei and extend to the sides of the upper tier, firstly transversely across the spindles and then vertically along secondary spindle fibres (Roy Chowdhury, 1962). The details of wall formation were not observed in this study, but photograph 36C shows that the upper nuclei are not separated from the egg cytoplasm by cell walls. This is true for most pines (Buchholz, 1931; Chamberlain, 1935; Doyle, 1963), but Thomas (1951) found that in many proembryos of P. virginiana the upper tier had formed complete cell walls and Dogra (1967) noted that this occurred as 'rare abnormalities' in pines.

Divisions in the two tiers of the primary proembryo in conifers result in a secondary proembryo (Dogra, 1967). In most conifers the upper tier divides to produce a secondary upper tier and a suspensor tier. The lower tier gives rise to the embryo initials. In pines, however, the upper tier divides to produce a secondary upper tier and a tier of cells



which has been called the rosette tier (in dissected embryos this group of cells has a rosette appearance). Doyle (1963) discarded this term and called the cells vestigial suspensors to indicate their relationship to the cells in other conifers (photographs 37B and D). In pines this division results in a twelve celled proembryo, but the lowest tier of four cells soon undergoes a second division and gives rise to four substitute suspensor cells (Dogra, 1967) and four embryo initials. Photograph 36D shows the division in the upper tier of the primary proembryo in P. radiata. After the division of the lower tier (photograph 36E) a sixteen celled proembryo is formed (photograph 36F). Elongation of the four substitute suspensor cells pushes the four embryo initials deep into the prothallus. Early elongation in P. radiata proembryos is shown in photograph 37A.

(f) The Corrosion Cavity and Endosperm. The corrosion cavity of pines has been described by Buchholz (1918), Sarvas (1962) and Dogra (1967). The development in P. radiata is typical. During proembryo development the prothallial cells below the archegonia develop dense cytoplasm and numerous starch grains. As the suspensors elongate (photographs 37A and 39B) these prothallial cells become loose and degenerate, and, as the embryos grow, a cavity is formed (photographs 37E and F). According to Dogra (1967), the embryos secrete enzymes into the corrosion region and absorb the products of the cells' breakdown. Buchholz (1918), however, noted that seeds without embryos still develop corrosion cavities. When embryo systems such as those in photographs 37B and D are dissected, the region surrounding the cavity is found to be tough, but the embryo systems can be pulled at the archegonial end and emerge from the cavity, which seems to contain viscous fluid. Sarvas (1962) observed that, in P. sylvestris, the archegonial walls break down to form a single cavity, which is continuous with the embryo cavity. He called this the primary corrosion cavity.

A gibberellin substance was found to increase in the seeds of

P. jeffreyi, P. ponderosa and P. lambertiana during the development of the young embryos, but it disappeared at seed maturity (Krugman, 1967).

While the embryo is developing the outer prothallial cells form many large globules of material, which stain bright red with safranin. As the embryos become more advanced these bodies increase in number in the outer cells and appear in the inner cells, until the remaining prothallus is uniform (photographs 37E, F, and 38A to D). This tissue is the endosperm of the seed. Konar (1958) showed that large amounts of reserve products, in the form of proteins, fats and starch, accumulate in the endosperm. At maturity the P. roxburghii embryo contains 31% fat (Konar 1958b).

(g) Embryo Development. After the substitute suspensor has pushed the embryo initials into the corrosion cavity, the embryo cells divide and the cells formed next to the substitute suspensors elongate as additional suspensors which terminate in embryo initials. The result is a complex of intertwining tubes, tipped with small dense cells (photographs 37B to D).

Once the embryo initials are thrust well into the chalazal end of the prothallus, they begin to divide, forming a clump of cells. Since embryo systems derived from several archegonia may be intertwined, some of the small embryo clumps will be of different genotype to others. The situation, in which there are several embryos in one seed, is termed polyembryony. This may be due to the fertilization of more than one egg, in which case it has been called simple polyembryony (Buchholz, 1920), polyzygotic polyembryony (Doyle, 1963) or archegonial polyembryony (Dogra, 1967). When the individual embryo systems develop separate embryo clumps from each embryo initial cell, the process is termed cleavage polyembryony. Photograph 37E shows several small embryos in the corrosion cavity of P. radiata. This photograph is very similar to one of P. lambertiana (Krugman, 1967). Only a small number of P. radiata seeds have been sectioned at this stage of development. The greatest number of

embryo clumps counted was eleven. Sarvas (1962) found that, in P. sylvestris, one to four embryo clumps occurred on each system and, at the most, eight embryo clumps were found in one seed. 45% of the seeds he observed contained three or four embryo clumps. Embryo clumps increase in size by cell division, but the size varies greatly between embryos, and eventually, one embryo pushes the other embryos and their suspensors to the micropylar end of the seed (photograph 37F and 38B to D). The competition of embryos is considered by some to be a selective mechanism (Sarvas, 1962). This will be discussed in greater detail in chapter VI.

As the embryo increases in size it differentiates distinct zones. The histogenesis and embryo organization in P. strobus has been described very fully by Spurr (1949). The structure of P. radiata embryos is very similar (photograph 38D). Towards the micropylar end of the seed the cells become arranged in files. Those nearest the micropyle are called the root cap cells. The centre cells from which the files appear to originate, are called the root initials and the column of cells above them is the hypocotyl zone of the young embryo. At the top of the embryo a small mound of thin walled, clear cells, the epicotyl, is flanked by mounds of cells with denser cytoplasm, the cotyledon primordia. Photographs 38A and C show an embryo at this stage of development. It may take two to three months after fertilization to reach this stage, although there appears to be a large between seed variation in developmental timing following fertilization. Having reached this stage, the cotyledon primordia increase in length until they fill the corrosion cavity (photograph 38D). The embryo is then morphologically mature, but may not be so physiologically. Even when it reaches physiological maturity (the time was not determined in this study) the seed remains dormant until it is released from the cone in conditions suitable for seed germination.

(h) Development of the Seed Coat. About the time the primary proembryo is being formed the middle/integument cells at the

micropylar end of the seed develop thick walls. The thickening gradually extends along the sides of the ovule and increases at the micropylar end. About the time that the substitute suspensors elongate the middle integument walls are very thick and perforated by many small pits (photographs 32G and H). Konar and Ramchandani<sup>(1958)</sup> have illustrated this for P. wallichiana.

The thickening of the middle integument walls apparently protects the seed at maturity. Kozlowski and Gentile (1959) showed that P. strobus seeds germinate more rapidly when they are punctured and the water and oxygen uptake by the seed are greatly enhanced if the seed coat is removed. Berlyn (1967) showed that in P. lambertiana dormancy of the seed was attributable to the seed coat.

Because the seed is released from the ovuliferous scale when the cone opens, the 'connecting' cells must form an abscission layer at seed maturity. In mature seeds there is a small raised point in the middle of the seed coat at the chalazal end. This may represent the scar of the 'connecting' tissue.

#### IV. DISCUSSION

In ovule morphology and embryogeny P. radiata is very similar to other species of Pinus. Occasionally small differences reported in some species, such as the absence of starch in year old pollen tubes of P. virginiana (Thomas, 1951), and the bulging of the nucleus of the egg at fertilization in P. roxburghii (Sethi, 1928), could have been due to fixing or staining techniques. Differences arising in the interpretation of the structure of the megaspore membrane could be resolved by a detailed T.E.M. study. Similarly, a T.E.M. study might show more clearly the relationship of the spongy cells to the megaspore and the jacket cells to the archegonium.

Features observed in P. radiata, which have not been commented on in the literature, may occur in other species. A definite band of cells passing through the ovule integument to the vascular tissue of the scale

was observed.

A study of the ultrastructure of these cells would be of special interest from several points of view. Their differentiation from the ovule toward the vascular tissue seems unusual. It is possible that these cells are specialized and are comparable to transfer cells described by Pate and Gunning (1972). Transfer cells are typified by numerous outgrowths of their cell walls, which develop relatively late in the life of the cell. They are thought to function in short distance transport of solutes and they occur in a variety of anatomical situations, in plants. These include the megagametophyte-sporophyte junction in flowering plants and the xylem parenchyma in pine seedlings (Pate and Gunning, 1972). The thickening of the 'connecting' cell walls about a year after they originate, their position in the ovule and probable function in providing a pathway for nutrient movement into the ovule suggest that they may be transfer cells.

The 'connecting' cells are also of interest in the light of the opinion expressed by Singh and Johri (1972) that although other gymnospermous ovules are vascularized, those of conifers are not, except for Cephalotaxus and Cedrus.

Although the structure and development of P. radiata ovules are comparable to those of other species, the time from pollination to embryo maturity (twenty months) is longer in P. radiata. Table 5:4 shows the developmental time for the few species for which the relevant data is given.

Species	Author	Duration
<u>P. Wallichiana</u>	Konar and Ramchandani (1958)	about 16 months
<u>P. roxburghii</u>	Konar (1962)	17 months
<u>P. sylvestris</u>	Sarvas (1962)	14 months
<u>P. siberica</u>	Nekrasova (1970)	14 months

Table 5:4 Time from pollination to morphological embryo maturity.

Table 5:5 summarizes developmental intervals in P.radiata and compares them with intervals given for other pines.

Species	Source	Interval	Duration
<u>P.radiata</u>	This study	polln.-meiosis	6- 10 weeks
other spp.	table 5:3	"	up to 3 wks.
<u>P.radiata</u>	this study	polln.- fert.	15 months
other spp.	table 5:1	"	11-13 months
<u>P.radiata</u>	this study	fert.- morph. embryo maturity	5 months
<u>P.wallichiana</u>	Konar and Ramchandani (1958)	"	about 5 mnths.
<u>P.roxburghii</u>	Konar (1962)	"	3- 4 months
<u>P.sylvestris</u>	Sarvas (1962)	"	embryo differ- entiates about 19 days.
<u>P.siberica</u>	Nekrasova (1970)	"	2 months

Table 5:5 Duration of developmental intervals in P.radiata and other species of Pinus.

From table 5:5 it appears that P.radiata takes longer than other species to develop at any stage. However, it is not clear whether the times given for embryo maturity in other species is for morphological or physiological embryo maturity. Although the time of any form of embryo maturity was not given by Thomas (1951) or Sarvas (1962), the rapid development in proembryo stages of P.virginiana and P.sylvestris suggest that the completion of embryo development morphologically is reached in a comparable time to that shown by P.siberica.

It has been suggested (G.B.Sweet, pers. comm., 1971) that the longer period of development in P.radiata is related to the closed nature of its cone, which enables it to remain on the tree for several years or more before seed dispersal. In species of Pinus which drop cones every year and especially in cold climates, the time available between fertilization and seed shed may be restricted. A study of other closed cone pines might determine whether longer cone development is associated.

## CHAPTER VI

FACTORS AFFECTING SEED PRODUCTION IN PINUS RADIATA

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## CHAPTER VI

FACTORS AFFECTING SEED PRODUCTION IN PINUS RADIATA

## I. INTRODUCTION

(1) Factors Affecting Seed Production

The final quantity of seed produced on a tree of P. radiata depends on a variety of factors. These are:

- a. number of cones initiated,
- b. extent of cone loss,
- c. number of potential seeds per cone,
- d. degree of failure during ovule and seed development.

The importance of each factor depends on site quality, weather conditions, genetic composition of the tree and probably other influences.

(2) Literature

The literature can be roughly divided into categories relating to the above factors. Only a few references are listed below because the subject of cone production has been fully reviewed by Puritch (1972) and Sweet (1973). The detailed study of seed production in P. sylvestris (Sarvas, 1962) covers many of the aspects considered in this study and describes much of the literature available prior to its publication.

Some relevant papers<sup>mainly</sup> published since 1962 are given here:

- a. records of seed crops: Bingham and Rehfeldt (1970); Bramlett (1972).
- b. records of seed yields per cone: P. radiata, Fielding (1964), Eldridge (1966), Burdon and Low (1971). Other species, Bergman (1968).
- c. cone losses: Frost damage, Hutchinson and Bramlett (1964). insects or squirrels, Hoekstra (1956) and Keen (1958) --neither reference given by Sarvas.
- d. cone abortion: Sweet and Thulin (1969), Sweet and Bollmann (1970), Brown (1971).



- e. ovule abortion: Bramlett (1971<sup>b</sup>)
- f. seed quality, embryo disturbance and the influence of selfing:  
Hagman and Mikkola (1963), Fowler (1964), Dogra (1967), Ehrenberg, Gustafsson, Plym Forshell and Simak (1967), Bramlett and Popham (1971)
- g. external influences: climate, Lester (1967), Vrendenburch and la Bastide (1969); fertilizer application, Barnes and Bengston (1958), Shoulders (1967); pruning or girdling, Bilan (1960), Ebell (1971)
- h. physiological aspects: Romberger (1967), Brondho (1970), Jackson and Sweet (1972)

### (3) The Scope of this Study

During the development of the female cone of P. radiata it was possible to obtain data on the number of seeds per cone for the clones studied, and on the number of ovule abortions. Causes of ovule abortion were investigated microscopically and data on pollen inviability, lack of pollination and lack of fertilization, recorded. Abortion types and their development were described, as well as other abnormalities. Some observations were made on early embryo break down.

## II. MATERIALS AND METHODS

### (1) Materials

Cone scale counts were made on cones of clones 19, 55, <sup>and</sup> 372, which were harvested twelve months or more after pollination. Such cones were harvested from Kaingaroa in 1968, 1970, 1971 and 1972 (clones 19 and 55) and from Whakarewarewa in 1971 and 1972. The sites have been described previously.

Microscopic sections of abortions were observed during the study of normal ovule development, and additional material was sectioned from the sectioned cones described above.

(2) Dissection

Cone scale counts were made by removing the ovuliferous scales, one by one, from fresh or preserved cones. The state of ovules (absent, rudimentary, normal, or aborting) was recorded at the same time.

## III. OBSERVATIONS AND RESULTS

(1) Total Number of Scales per Cone

In P. radiata, as in other species of Pinus, (Lyons, 1956; Sarvas, 1962; Bramlett, 1971b) the cone can be divided into three zones. The scales of the lowermost do not bear ovules. Between this zone and the zone of normal ovule bearing scales there is a region of scales bearing rudimentary ovules. These consist of integument cells and a clump of inner cells. The integument cells in the region of the micropylar arms swell at cone closure and may even enclose a pollen grain in the micropyle of the rudimentary ovule. But, rudimentary ovules never from a megaspore mother cell and the integument and nucellus cells do not continue growth for more than a few months after pollination. A rudimentary ovule of P. radiata is illustrated in photograph 39A. In chapter IV, section 7, observations and results, it was shown that the lowermost ovules in the <sup>normal</sup> ovule bearing zone are smaller than the other ovules and tend to accommodate fewer pollen grains. Nevertheless, they produce a megaspore mother cell, and providing they are pollinated, develop into normal seeds.

Therefore, it is the number of scales bearing normal ovules, which gives the number of potential seeds per cone. However, when the number of scales bearing normal ovules is plotted against the total number of scales per cone (figures 6:1 and 6:2), a linear relationship can be seen. A similar figure is given by Lyons (1956). Therefore, data obtained from counts of the total number of scales per cone is relevant as a factor affecting seed production. The mean number of scales per cone is given in table 6:1.

Initiation		Clone 19	Clone 55	Clone 372
Year				
site				
K	1967	183.1	182.7	-
A				
I	1969	171.6	205.1	-
N				
G	1971	204.7	201.0	-
A				
R	1972	211.5	214.7	-
O				
A				
W	1971	192.0	169.7	179.3
H				
A	1972	217.6	212.8	185.3
K				
A				

Table 6:1 The mean number of scales per cone.

Because more years were sampled at the Kaingaroa site and one more clone was sampled at Whaka, several analyses of variance were done.

These were: a. Kaingaroa (four years, two clones)

b. Whaka (two years, three clones)

c. Both sites (Two years, two clones)

d. Kaingaroa (one year, one clone, two trees).

To avoid repetition in the following text each analysis will be referred to by the corresponding letter (a, b, c or d). Analyses of variance are summarized in appendix 6.

In the analyses of clones 19 and 55, the clonal differences were small (5% in a, NS in c). But, in analysis b, clone 372 had fewer scales than clones 19 and 55 (significant difference at 0.1% level). Between two trees, one on the edge of the stand and one surrounded by other trees, no significant difference in the total number of scales was detected. Because conditions on the edge of the stand are probably different from those within the stand (more light and exposure and less competition from other trees) a difference detected within a clone would mean that the character being investigated (in this case total scale number) per cone may be environmentally rather than genetically determined. The fact that within <sup>1</sup>clone differences were not detected in

these conditions means that clonal comparisons are justified.

In all analyses year influences accounted for most of the variation in total scale number, but a small site difference (5% level) was also detected in analysis c.

A significant year-site interaction (1% level) suggests that in any year there are different climatic conditions at the two sites, but could also reflect the difference in age of the trees at the two sites. A year-clone interaction at each site (5% in a, 0.1% in b) suggests that clones vary in their response to different climatic conditions.

Burdon and Low (1971) looked at scale numbers at four sites and found significant differences between Whaka and Berwick, the sites with extreme means. These authors did not investigate clone or year influences on scale number. The mean number for the three clones at Whaka in two years was lower (196.7) in this study than the mean for eighteen clones at Whaka, studied by Burdon and Low (209).

## (2) Potential Number of Seeds

It has been shown that the number of scales bearing normal ovules increases as the total number of scales increases. However, for each site the regressions of clones 19 and 55 were compared using a t-test, and were found to differ significantly (0.1% level). Site differences were shown for clone 55 (0.1% level) and clone 19 (5% level). At Whaka the regression for clone 372 was markedly different from the other clones, suggesting that the relationship can be influenced by genotype. Therefore, genotypic and site factors, because they affect the number and proportion of scales bearing normal ovules, influence the potential number of seeds (number of scales bearing normal ovules  $\times 2$  ).

Table 6:2 gives the sample means for potential seed number. Analyses of variance are given in appendix 7A to D. They show highly significant differences in potential ovule numbers between clones (0.1% level). Significant site, and year differences (at Kaingaroa), were also detected (1% and 5% levels, respectively). A significant clone-year

interaction at Kaingaroa and Whaka (1% and 5% levels) shows differences in clone response to weather influences, and a year- site interaction (0.1% level) was, again, detected (appendix 7D).

	Initiation Year	Clone 19	Clone 55	Clone 372
site				
K	1967	167.8	139.2	-
A	1969	150.2	144.0	-
i				
N	1971	185.6	140.9	-
G				
A	1972	172.2	133.2	-
R				
O				
A				
W	1971	158.0	111.1	144.7
H				
A	1972	173.8	133.6	135.1
K				
A				

Table 6:2 The mean number of potential ovules per cone.

The number of scales bearing normal ovules, calculated as a percentage of the total cone scales, ranges in clones 19 and 55 from 36 to 54% and from 28 to 42% respectively. Therefore, clone 19 cones generally have more potential seed than clone 55 cones, despite the fact that the mean total number of scales is similar. Although clone 372 has fewer scales in all, the mean percentage of ovule bearing scales (38%) lies between those for clone 19 and 55 (41.4% and 31.5% respectively). However, clone 372 is more variable and its range (28 to 49%) overlaps the ranges of clones 19 and 55.

Since the potential number of seeds is modified during the subsequent development, it is not justifiable to exclude 'low seed potential' clones from the seed orchard without further investigation.

### (3) First Year Ovule Abortion

The growth of ovules which abort during the first year is arrested soon after meiosis and a few months later they can be distinguished visually from normal ovules by their size difference.

Species & author	Site	Year	% 1st. year abs.	% 2nd. year abs.	% seed abort- ions.
<u>P. resinosa</u>	Sault Ste. Marie	1951	27	NR	10
Lyons, 1956	Camp Borden	1950	42	"	NR
	"	1953	41	"	12
	Chalk River	1950	37	"	18
	"	1952	38	"	NR
	mean		37		13
<u>P. sylvestris</u>	Tusula	1959	23	"	14
Sarvas, 1962	Sippola	"	26	"	11
	Padasjoki	"	23	"	14
	Kerimaki XX	"	17	"	14
	Kerimaki XXXIII	1958	13	"	13
	"	1959	24	"	17
	Vilppula	"	24	"	13
	Kuorevesi	1958	19	"	12
		1959	28	"	15
	Roveniemi mlk.	1958	35	"	14
		1959	48	"	12
	Roveniemi	1958	30	"	14
	Inari	"	54	"	17
		1959	31	"	16
	mean		28		14
<u>P. echinata</u>	Buckingham,	1966	15	59	19
Bramlett, 1971	Virginia.	1967	28	56	12
	"	1968	31	58	9
	"	1969	18	28	24
	mean		23	50	16
<u>P. siberica</u> <small>NEKRASOVA (1970)</small>	Urals, U.S.S.R.	1966	46	NR	3

Table 6:3 Percentages of different kinds of ovule and seed loss, calculated from data given in the literature for some species.

From data in the literature, summarized in table 6:3, it appears that in other species abortions of ovules in first year cones account for high reductions in the potential seed yield. Data for some species have been obtained from different sites and years, but no previous investigations on the number of abortions in different clones have been carried out. Burdon and Low (1971) considered that they had indirect evidence that abortions are prevalent in P. radiata but had no direct counts.

Table 6:4 shows the mean number of abortions per cone expressed as a percentage of the mean number of potential ovules in that sample.

Initiation Year		Clone 19	Clone 55	Clone 372
site K A I N G A R O A	1967	12.9	4.4	-
	1969	13.5	10.0	-
	1971	21.3	5.4	-
	1972	10.8	10.2	-
W H A K A	1971	28.2	25.9	18.8
	1972	8.8	3.0	8.5

Table 6:4 The mean percentage of abortions per year old cone.

Analyses of variance of the transformed (angular) percentage are given in appendix 8a to d. These showed a clonal effect (0.1% level) for clones 19 and 55, but when clone 372 was added (analysis b) the clonal difference was reduced to 5% level. In analysis 8b and c a highly significant year affect was detected (0.1% level). No site difference was shown but a year-site interaction was again detected (0.1% level). At each site (analyses a and b) there was a clone-year interaction (1% level) but no between tree differences were shown (analysis d).

The results show some abortion levels which are comparable to

those of other species (table 6:3), but usually abortion losses in P.radiata are not as large. The causes of first year abortions of P.radiata and their frequency will be considered in sections 5 and 6.

(4) Net Production of Viable Ovules at the end of the First Year's Development

In previous sections it was shown that clone 55 has a lower seed potential than clone 19, but also has a lower percentage of first year abortions. Clone 372 lies between the two in both respects. In these clones, therefore, the two factors tend to balance one another in their influence on the number of viable ovules per cone. Since the number of seeds produced is dependent on the number of viable ovules at the end of the first year, it is useful to analyse the data statistically. In table 6:5 the mean number of viable ovules per cone is given.

	Initiation Year	Clone 19	Clone 55	Clone 372
site				
K	1967	142.3	125.0	-
A				
I	1969	131.4	130.3	-
N				
G	1971	145.0	132.0	-
A				
R	1972	155.4	119.4	-
Q				
A				
W				
H	1971	112.2	81.9	117.9
A				
K	1972	155.1	129.2	123.1
A				

Table 6:5 Mean number of viable ovules per cone.

Analyses of variance are given in appendix 9a to d. In all cases there were significant clonal differences (1% level in 9a and b, 0.1% level in 9c) and in all except a, a year effect was shown (0.1% level). The effect of site and the year-site interaction were both significant (0.1% level). Only in 9b was a year-clone interaction shown (1% level) and, once again, there was no significant difference between grafts of



clone 55, growing in different parts of the orchard.

Analysis of the number of viable ovules at the end of the first year's development gives the net effect of factors investigated in previous sections.

#### (5) Microscopic Appearance of Aborting Ovules

In section 3 it was shown that <sup>ovule</sup> abortions may account for a reduction of 20% or more in the potential seed number, although in some clones, years and sites this loss may be much lower. In considering the causes of ovule abortion it is necessary to describe the different types: their morphological appearance and development.

Two kinds of ovule abortion were identified. These were the first year ovule abortions counted by dissection (section 3) and second year ovule abortions- ovules which degenerate after fertilization and, until then, cannot be separated visually, with any certainty, from normal ovules. Both kinds develop apparently normal seed wings, although, in first year ovule abortions they may be smaller than in normal ovules.

First year aborted ovules may or may not be pollinated. The frequency of each will be discussed in a later section. In aborted ovules which are pollinated, the grains usually have not germinated. First year aborted ovules fail to develop starch grains in the cells of the nucellus cap in the post pollination period, when it becomes abundant in normal ovules. (photograph 39C). About the time of meiosis the spongy tissue cells enlarge and often contain starch grains. The megaspore disappears and the megaspore region becomes filled with spongy cells (photographs 39D and E). These cells progressively degenerate, forming a deeply staining amorphous mass, interspersed with some remaining spongy cells. Frequently, following this, the nucellus cells just below the cap degenerate into a band of collapsed cells (photographs 40A, B and C). About a year after pollination the whole nucellus withers (photograph 40D). Throughout the development of the aborted ovule, up to the time of fertilization in the cone, the integument cells look

normal, but the integument does not grow at the usual rate. The aborted ovules, ten months after abortion, are only one third the length of normal ovules. Figure 6:3 shows that they do not reach more than 1.5 mm in length. The size difference between a normal ovule and a first year aborted ovule can be seen in photographs 40B and 41A. When fertilization is occurring in other ovules the integument cells in aborted ovules become sclerified and then degenerate (photograph 41C). From this stage onward the aborted ovule's cells probably cease to absorb nutrients from the rest of the cone.

Pollen germination and growth in ovules which abort in the second year is apparently normal for the first year, even though the megaspore is absent. Therefore, the megaspore probably does not influence the growth of pollen within ovules. However, second year aborted ovules probably do not result from failure of the megaspore to develop at meiosis because all three hundred ovules (excluding first year aborted ovules), observed during the month following meiosis, had megaspores. But, in mid December (about six weeks after meiosis) three pollinated ovules appeared to be lacking a megaspore, and their spongy cell walls looked slightly thicker than usual. A small number of ovules harvested at the beginning of January were clearly recognizable as second year aborting ovules; they had no megaspore, a collapsed megaspore or a few degenerating cells in the centre and the spongy cells were thick walled with dense cytoplasm. In later harvests these features became more distinct (photograph 41D), but other tissues, including the 'connecting' cells, developed normally.

From this it seems that megaspore breakdown occurs before the megaspore nucleus divides, at a time when embryo growth in the previous year's cones is just beginning. Whether the first year cones suffer nutrient stress, which could cause physiological competition between ovules, cannot be shown. The cause of second year abortions is probably related to physiological and/ or genetic factors.

By September the modified cells of the spongy region in the second

year aborting ovules begin to degenerate and their nuclei become distorted (photograph 42A). Photograph 42B shows that such ovules are all slightly smaller than normal ovules and the dimensions of each can be seen from figure 6:3. It is possible that these abortions would be classified as empty seed, but as figure 6:3 shows, they are about half the length of normal seeds, once seed maturity is reached. Photograph 42C shows a second year aborted ovule beside an edge section of a normal seed.

First year aborting ovules with enlarging spongy cells have been described by Ferguson (1904) and Konar (1960, 1962), but neither author related their occurrence to a lack of pollination. Sarvas (1962) describes these aborted ovules in great detail and shows that unpollinated ovules fail to develop normally. In P. sylvestris (Sarvas, 1962) the megaspore degenerates twelve days after meiosis. Sarvas does not record instances of first year aborted ovules which are pollinated.

First and second year aborted ovules are described by Lyons (1956), but his criterion of the latter is that they lack archegonia and he does not describe their appearance.

#### (6) Frequency of Abortion Types

Table 6:6 shows that, at Kaingaroa, most of the first year aborted ovules of clone 19 were not pollinated and the number of pollinated first and second year aborted ovules were often about the same. In clone 55 the number of unpollinated aborted ovules was much lower, but the number of pollinated aborted ovules, particularly the first year aborted ovules, was higher. Therefore, the reduced number of aborted ovules shown for clone 55 in section 3 results from better pollination in this clone.

The percentage of aborted ovules calculated from sections of about thirty ovules (table 6:6) often differs considerably from the percentage obtained by dissecting whole cones from the same clone, year and site. Percentages of aborted ovules in table 6:6 include the contributions

from second year abortions, but are, nevertheless, in some years, lower than in table 6:3. This must be put down to the relatively small sample size ( thirty ovules), when there are often only a few aborted ovules in the whole cone. Therefore, the results in table 6:6 are rough estimates; their importance lies in the relationships between the different types of aborted ovules.

KAINGAROA					COMPOSITION OF ABORTED OVS.		
clone	year	raw data		% Abtd	1st year		2nd year
		No.ovs.	No.Abtd		% unpoll'd	% poll'd	
19	1967	90	24	26.7	91.7	0.0	8.3
	1968	162	25	15.4	72.0	12.0	16.0
	1969	224	15	7.8	60.0	13.3	26.7
	1970	281	12	4.3	66.7	16.7	16.7
	mean				75.0	9.2	15.8
Type of aborted ovule as % of total					7.5	0.9	1.6
55	1967	178	5	2.8	0.0	40.0	60.0
	1968	129	14	10.9	0.0	21.4	78.6
	1969	234	18	7.7	5.6	61.1	33.3
	1970	267	57	19.1	21.1	56.1	22.8
	mean				13.8	51.1	35.1
Type of aborted ovule as % of total					11.6	5.9	4.1
WHAKA							
19	1971	287	57	19.8	84.2	14.0	1.8
55	1971	257	25	9.7	80.0	16.0	4.0
274	1971	148	43	29.1	81.4	7.0	11.6
372	1971	306	28	9.2	17.9	64.3	17.9
	1972	154	31	20.1	61.3	32.3	6.5
	mean				40.7	47.5	11.9
Type of aborted ovule as % of total					5.2	6.3	1.5

Table 6:6 The frequency of aborted ovules and the proportion of each type of aborted ovule, expressed as a percentage of the total number of aborted ovules and the total observed ovs.

A higher percentage of pollinated aborted ovules occur in clone 55 at Kaingaroa, usually because the pollen grains have failed to germinate. Often such ovules contain more than one pollen grain which has failed. Such failure may be due to a high proportion of inviable pollen grains arriving in the cone, or to an unfavorable ovule-pollen interaction. A very small proportion of ovules at the tip of the cone in any clone fail to develop for lack of space (photograph 39B). A study of the frequency of pollen inviability could be related to the number of pollinated aborted ovules, and will be described in section 7.

Table 6:6 shows that the proportion of ovules which become second year aborted ovules is relatively low and is not very different between the clones. It probably would not affect seed production greatly.

#### (7) Pollen Inviability

Pollen inviability was determined for individual pollen grains, from microscope sections of ovules at all stages following pollen germination. Grains which had failed to germinate were scored as being inviable; they usually looked misshapen and had densely staining contents. (photographs 31, 40C and D). In thirty ovules there may be twenty to a hundred and sixty pollen grains in the micropyles, so the sample sizes per cone varied greatly. Pollen inviability was therefore calculated as a percentage of the total in each cone. In general, the percentage was low, around 5% inviable, but in one cone from clone 55, 1970 it reached 55% and was very high in four out of six cones from this harvest. Table 6:7 shows the mean percentage of inviable pollen grains from cones and ovules sampled each year. Because the distribution of data was skewed angular transformation of the data was carried out before statistical analysis.

Table 6:7 Mean percentage of inviable pollen grains in cones from Kai.

Clone	1967	1968	1969	1970	1971
19	8.6	4.4	7.5	3.8	5.4
55	6.1	5.6	13.6	24.5	7.7

This method of measuring the final pollen inviability does not detect the possible causes. Inviabile pollen could be caused by abnormal behaviour of the chromosomes at meiosis in the pollen mother cell, or to nutritional or temperature stress at the time of pollen formation. Rain is known to damage pollen (Sarvas, 1962), but pollen on the trees is probably protected from water by the scales of the male cone and, since pollen in flight is probably carried to the ground in raindrops, rain damaged pollen is unlikely to reach the female cones. A third cause of pollen failure to germinate could come from an ovule- pollen interaction, either a genotypic interaction ( which seems unlikely because some pollen from other species of Pinus can germinate on the nucellus), or abnormal physiological behaviour on the part of the ovule in cases where all of the several grains in a micropyle fail to germinate.

Two factor analysis of variance of the percentage of inviable pollen was carried out (appendix 10). This detected a significant between clone and clone- year interaction (1% level). A significant between year difference was detected at the 5% level. Clone 55, which has the most pollinated abortions, produced many ovules in which multiple germination failures were present. These accentuated the pollen inviability count. However, the number of non pollinated ovules in clone 19 was so high that it still had significantly more aborted ovules than clone 55, inspite of the effect of the number of pollen grains which fail to germinate in the latter.

#### (8) Failure at Fertilization

Virtually all ovules which do not abort develop at least one archegonium and accommodate at least one pollen grain to fertilize it. The number of archegonia in each ovule and those which were fertilized was recorded. The data are summarized in table 6:8, for cones harvested after mid November, when fertilized eggs contained proembryos or developing embryos. Non fertilized eggs were distinguished by their large

central, single nucleus, dense cytoplasm, closed neck cells and intact ventral canal cell wall. Photographs 43A to D show fertilized eggs at various stages and unfertilized eggs adjacent to them. As normal embryo development proceeds the egg cytoplasm is broken down and absorbed by the embryo. Egg cytoplasm degeneration in unfertilized eggs is retarded so there is a contrast in the density of the cytoplasm of fertilized and unfertilized eggs.

Site & Clone		Date	0/1	1/1	0/2	1/2	2/2	0/3	1/3	2/3	3/3	0/4	1/4	2/4	3/4	4/4
K	55	12-12-68		1			25			12	21		1	1	3	1
K	55	30-11-70				7	14		6	4	7					
W	55	16-11-72			1	3	3		6	7	7			1		
W	55	16-11-72				2	5		5	6	4		1		1	
Total			0	1	1	12	47	0	17	29	39	0	2	2	4	1
K	19	12-12-68		2		10	8		1	5						
K	19	12-12-68		6	5	19	7		2							
W	19	16-11-72				6	13			3	2					
W	19	16-11-72	1	4	4	13	5		1							
Total			1	12	9	48	33	0	4	8	2					
W	372	16-11-72		5		9	8		3	2	1					
W	372	16-11-72		3	1	7	5	1	2	3						
Total			0	8	1	16	13	1	5	5	1					
% ovules with 1, 2 etc. arch.			6.8		55.9		34.5		2.8							

Table 6:8 The frequency of ovules with 0, 1, 2... fertilized eggs over the total number of eggs in the seed.

Table 6:8 shows that the frequency of single archegonia (= eggs) is low. Therefore, the potential for polyzygotic polyembryony in P. radiata is high. The most common reason for unfertilized eggs is a lower number of pollen grains than archegonia in an ovule. This does

not affect the development of the seed since the pollen present is usually successful, but in a small proportion of ovules all the eggs in one ovule may be unfertilized because the pollen grows abnormally. This is one cause of empty seed (table 6:9).

Site	Frequency of failures			% Failures		
	55	19	372	55	19	372
Kaingaroa	1/122	4/ 86	-	0.82	4.65	--
Whaka	3/231	6/212	0/121	1.30	2.83	0.00

Table 6:9      The number and percentage of fertilization failures in seeds harvested at post fertilization stages. Sample sizes vary because data from material used for embryo dissections is included.

Photograph 46B shows a degenerating seed with withered prothallus resulting from complete failure at fertilization or soon afterwards.

The relationship of archegonial number to the number of fertilized eggs is also of interest to tree breeders for it indicates the frequency of polyzygotic embryos and the number of genotypes available for 'selection' of the surviving embryo. The clonal difference in archegonial number is marked. In clone 55 60% of the ovules contain more than two archegonia; in clones 19 and 372 12% and 24%, respectively, contain more than two archegonia. However, in clone 55, the average number of pollen grains accommodated in each ovule is also much higher so that 91.6% of the developing seeds contain more than one proembryo. In clone 19 only 66.2% of the ovules contain more than one embryo which explains why there is a higher percentage of fertilization failures (table 6:9). There is also reduced opportunity for genotypic selection. If a single embryo contains a homozygous lethal gene and fails to develop, it will not be supplanted by a normal embryo and again, empty seed will result.



In table 6:10 the number of pollen grains and the number of archegonia in each ovule from a cone of clones 19 and 55 from Whaka are given.

19					55			
Number of pollen grains in micropyle.								
No. arch.	1	2	3	4	1	2	3	4
1	1							
2	4	6	9	1	3	2	2	2
3		2	2		7	5	2	5
4						1		

Table 6:10 The frequency of ovules with x pollen grains and y archegonia.

The variety of combinations of variables given in table 6:10 suggests that there is no distinct within ovule relationship between pollen grain number and the number of archegonia formed, even though clone 55 has more pollen grains per ovule and more archegonia per cone.

Abnormal behaviour on the part of the pollen grain may be of several kinds. In some cases the pollen fails to resume growth in the second year. Occasionally pollen tubes grow but not towards the archegonial necks. There might be the same number of pollen tubes as archegonia but one may penetrated the prothallial tissue alongside an archegonium. In one cone of clone 372 (1972) a pollen tube in a normal ovule was found to branch into two archegonial necks, but sperm nuclei and cytoplasm passed into only one egg. In a cone of clone 19 (Whaka, 1972) three different ovules contained pollen tubes which had passed into the archegonial depression but had not injected their contents into the eggs. Degenerating cytoplasm and sperm nuclei were seen in the tube in the archegonial depression, but other eggs in these ovules had been successfully fertilized. This source of failure was found in only one cone, several times, which suggests that the expelling of pollen contents into the egg may be influenced by a stimulus that was rather weak in this cone. If this

is so, then fertilization is not necessarily a consequence of pollen tube growth.

In most of the material studied pollen inviability and failure of pollen to fertilize available eggs did not affect the seed production. Sarvas (1962) concluded that pollen inviability and failure at fertilization in P.sylvestris is generally low because the percentage of fertilized eggs was very close to the percentage expected from the pollen levels.

#### (9) Embryo Breakdown

Evidence of embryo breakdown in early stages of development in P.radiata is shown in photographs 44B to D, which illustrate some of the different types encountered. Photograph 44A shows two normally developing embryos lying side by side, but one has been thrust more deeply into the corrosion cavity because it either began to elongate earlier or elongated faster than the other embryo. In photograph 44B the more advanced embryo has obstructed the other, resulting in the latter's degeneration. Photograph 44C shows a normal and an aborted embryo prior to elongation of the substitute suspensor in either. In photograph 44D two normal embryos have elongated but neither is in contact with the third, aborted embryo. The fertilized ovule in photograph 43C was also aborting, even though the other archegonium was not fertilized. Therefore, although some embryos appear to be actively competing at the time of elongation of the substitute suspensors, this is not always the case and embryos may fail in the absence of obvious competition. These failures could be due to the presence of homozygous lethal genes in the embryo (Sarvas, 1962). One elongating embryo was observed to be curling back into its archegonial cavity rather than elongating into the corrosion cavity. However, embryo failure prior to, or at the time of suspensor elongation in P.radiata, is not frequent. The number of such embryos recorded for the material observed is shown in table 6:11.

Clone Site	Date of Harvest	Number of degenerating embryos	Number of embryos observed
K 55	12-12-68	0	139
K 55	30-11-70	1	72
W 55	16-11-72	1	40
W 55	16-11-72	3	45
W 19	16-11-72	3	45
W 372	16-11-72	1	40
W 372	16-11-72	1	28

Table 6:11      The number of degenerating embryos identified in immature seeds of P. radiata.

In section 8 it was shown that 91% of immature seeds of clone 55 and 66% of immature seeds of clone 19 contain more than one embryo. Yet it appears to be rare for mature seeds to do so. Figures cannot be given here because only a small number of mature seeds have been cut open and none with double embryos were found, but there is usually no mention in the literature of double embryos in Pinus species. This means that a large number of immature embryos must fail to continue development at a later stage. Following the stage of embryo development shown in photographs 37B to D only a small number of seeds were sectioned. These seeds contained closely entwined embryo systems with scattered embryo clumps. It was impossible to determine whether these clumps were from different eggs or not. Usually the embryo which was deepest in the cavity was largest and the effect probably would become more noticeable with time, but since harvests made after fertilization were a month apart, the trend could not be observed in this study. Although the leading embryo might survive because of its position and may even inhibit the growth of other embryos, as Mikkola (1969) suggested, it could have achieved this position by competition at the time the substitute suspensors elongated.

# (10) Other Abnormalities

Other abnormalities which were observed during the study were very rare and probably do not affect seed production because viable embryos can still be produced by the developing seed, despite the occurrence of one of these abnormalities within it.

In chapter V the regularity of pollen germination in the micropyle and penetration of the nucellus was reported. Photographs 45A and B show two pollen grains in different cones of clone 7, 1970, which were seen to penetrate micropylar arm tissues. Their growth, however, did not proceed far. In two cones of clone 55, harvested in 1970, about a year after pollination, pollen had germinated but had not penetrated the nucellus cap. They had grown over its surface instead. In both ovules the megaspore had failed to develop. On the other hand, one ovule was found to have developed normally a year after pollination, even though there was no pollen present in the micropyle.

In the course of this study more than 2,800 ovules were observed after megaspore formation had occurred. Two cases of double megaspores were found, one in an early stage of development (photograph 46A) and one with two prothalli (photograph 46C). In P.virginiana (Thomas, 1951) the frequency of two gametes was reported as being higher than in other species of Pinus. Thomas considered that each megaspore was a product of the diad formed at meiosis. She found that double gametophytes were retarded in their development. This may also apply to P.radiata as fertilization of the eggs in each of the prothalli had not occurred, although other seeds in the cone contained young embryos. Ferguson (1904) also reported the incidence of double megaspores as well as occurrences of archegonial proliferation. Sethi (1928) found a few prothalli bearing abnormally high numbers of archegonia, but this phenomenon was not observed in P.radiata. Double megaspores occur in about 1% of the P.sylvestris ovules in Finland, but there was some evidence that their incidence

was affected by genotypic factors (Sarvas, 1962).

#### IV. DISCUSSION

##### (1) Seed Production in the Clones Studied

The results summarized in table 6:12 show that clone 19 had the greatest number of potential seeds and produced more mature seeds per cone.

Total scales	Site	19	55	372
	K	19269	20124	-----
	W	20550	19240	18300
Potential ovules	K	16900	13780	-----
	W	16630	12290	13870
No. viable ovules in first year	K	14350	12668	-----
	W	13480	10680	12120
Potential no. seeds at fertilization	K	140796	12105	-----
	W	13412	10642	11910
No. viable seeds after fertilization	K	13425	12006	-----
	W	13032	10504	11910

Table 6:12 Mean number of ovules and seeds in a hundred cones from each clone and site at each stage of cone development.

Clone 55 had fewer potential seeds (74 to 82% of clone 19) and produced fewer surviving seeds (81 to 89% of clone 19). Clone 372 lies between the other clones in both respects.

Although a reduction of 13 to 20% of the potential seed in the clones studied was due to failures of various kinds, it appears that the number of normal ovules initiated is an important factor affecting seed production. The number is influenced by the total number of scales in the cone and by the genetic ratio of normal ovule bearing scales to

total scales. The total number of scales is, in turn, influenced by the genotype and the environmental conditions of year and site. Site differences detected could be due to the difference in age of grafts. Cones dissected in 1971 from Whaka, were initiated almost two years after the establishment of the clonal area, while those at Kaingaroa were initiated thirteen years after the grafts were transplanted. The main climatic differences between the two sites would be due to differences in altitude, but edaphic conditions, since both sites are situated on the volcanic plateau, would probably be similar.

This study was limited to the seed production of cone samples. In considering the clonal contribution to seed production in a seed orchard it is equally important to study the number of cones per tree. This aspect could not be investigated in this study, but some measure is given by the number of ramets supplying cones for field observations in 1972. These are summarized in table 6:13.

Clone	No. ramets	No. cones	Cones not observed
19	3	20	2 cycles on each leader
55	1	90	3 cycles on leader and a few on high branches.
372	2	78	2 cycles on each leader and a few cones on high branches of tree two.

Table 6:13      An estimate of the number of cones produced by the clones observed at Whaka in 1972.

This shows that clone 55 produces many more cones per tree than clone 19, so despite its smaller number of seeds per cone, in terms of total seed production it is probably more useful in a seed orchard. Furthermore, the seeds of clone 55 were generally larger than those of clone 19 and may therefore produce larger and possibly more vigorous seedlings. Because of their higher frequency of polyzygotic embryos, there

is probably only a small number of empty seeds resulting from embryo failure, a second factor which could not be fully investigated in this study. If a 'selection' mechanism operates on polyzygotic embryos, clone 55 seed, because it contains more genotypes for selection overall, may be of better quality and the germination percentage higher than in other clones.

Although this study does not present the complete picture of seed production in the observed clones, it indicates that clones with a lower number of seeds per cone may have other features which favour their inclusion in a seed orchard.

## (2) Abortion of Ovules in *Pinus radiata*

Although the influence of aborted ovules on seed production in *P. radiata* may not be as great as in other species, the study of aborted ovules has yielded some interesting points, which are summarized below:

- (i) abortions are not necessarily due to pollination failure
- (ii) pollination failure is predominant in some clones only
- (iii) in *P. radiata* there are two types of abortion which are pollinated.

Circumstances<sup>s</sup> of pollination failure in *Pinus*, described in the literature, are various. Sarvas (1962) related abortion percentages in *P. sylvestris* to low pollen production in the stands. Nekrasova (1970) considered that aborted ovules in *P. siberica* were caused by lack of pollination due to late cone receptivity, or to low pollen production in a dry spring. Brown (1971) found that 25% of control pollinated *P. sylvestris* ovules contained no pollen, even though plenty had entered the cones

In chapter IV, from the quantity of pollen trapped in naturally pollinated cones of *P. radiata*, it was concluded that in conditions of natural pollination at Kaingaroa and Whaka, there was usually enough pollen in the air for atleast one pollen grain to reach every ovule. Data on the number of unpollinated ovules in clone 19 suggest that this

was not always so. One explanation of the apparent contradiction is that pollen is not randomly distributed in the cone, or, if it is, it is not always successfully transported into the micropyle.

Analysis of data on pollen trapped (section 4, observations and results, chapter IV) showed that clone 19 usually traps less pollen than clone 55, and there is less pollen trapped at Whaka than at Kaingaroa. In this chapter it was shown that clone 19 has more unpollinated ovules and that both clones had an increased percentage of unpollinated ovules at the Whaka site (table 6:6). In 1971 clone 372 trapped more pollen on average than other clones at Whaka and also had a lower percentage of unpollinated ovules. However, in 1972 cones of clone 372 generally trapped more pollen than in 1971, but the proportion of unpollinated ovules was higher. The correspondence between the amount of pollen trapped and the number of aborted ovules is not really clear, but might have been shown if more cones had been sampled.

If pollen is not randomly distributed in cones it would require a higher density of pollen to supply all ovules. Sarvas (1962) has investigated the distribution of aborted ovules within cones and has shown that a higher frequency of unpollinated aborted ovules occurs on the side away from the prevailing wind. Bramlett (1971) has suggested that the ovoid shape of the cone means that the lowermost ovule bearing scales are less accessible to airborne pollen. Because the receptive duration of most P. radiata cones is longer than in P. sylvestris, and because there is a clonal difference in the number of unpollinated aborted ovules, wind direction may not contribute greatly to the number of unpollinated ovules of P. radiata. Clonal cone shape could account for the clonal difference, but, if so, it would be expected that almost all aborted ovules, which are unpollinated, would occur in the upper and lower zones of the scales which bear normal ovules. In most cones of clone 19 there are unpollinated aborted ovules scattered throughout. <sup>Four</sup> ~~Three~~ possible



explanations for the clonal relationship of unpollinated ovules can be suggested:

- (i) Some ovules in clone 19 may fail to produce a pollination drop, or may produce a small one which does not reach the micropylar arms.
- (ii) Early needle elongation could obstruct some scales of the cone so that pollen cannot easily pass between them, but usually cones are well clear of the needles during pollination.
- (iii) The small size of the cone in some clones could mean that the between scale channels are so narrow that they limit pollen arrival.
- (iv) The duration of receptivity of the cones of some clones may be very short in some pollination years, because clonal receptivity could coincide with a period of wet weather.

The abortion of pollinated ovules is usually associated with the failure of pollen to germinate. When pollen viability is high, it would not affect the seed production, because only a few ovules would receive single pollen grains which were inviable. It would be expected that this situation would occur more frequently in clone 19 in which the frequency of single pollen grains is higher. In fact, the percentage of pollinated aborted ovules in clone 55 was six times that of clone 19. It is difficult to explain this difference in terms of pollen viability within cones of different clones. If pollen inviability is caused by a failure during pollen development its occurrence should not be related to the genotype of the female cones. Pollen clouds of different composition would reach receptive cones at different times. Differences in pollen composition in the micropyles could be related to clonal receptivity, but, usually, clonal receptivity of one clone overlaps that of another so the differences in pollen composition would not be so marked. In 1970 pollen inviability in clone 19 was one eighth that of clone 55; a dif-

ference which seems too great to be explained by differences in the pollen clouds. It might be suggested that a high degree of selfing could account for the failure of pollen in clone 55, but at Whaka clone 55 produces very little pollen. A pilot experiment produced no evidence of reduced pollen germination in selfpollinated cones of clone 89.

Possibly the high level of pollen inviability in clone 55 is due to the influence of the ovule on the pollen at the time of pollen germination. This would involve a physiological explanation which is beyond the scope of this study. Lyons (1956) does not consider the influence of pollen as a cause of first year ovule abortion, but suggests the role of ovule nutrition as a cause.

The cause of second year ovule abortion is possibly a physiological one also. The absence of information in the literature on second year aborted ovules of the type described here may be due to their infrequent occurrence in other species. It is possible that they have been overlooked, because previous work on aborted ovules was based on their external appearances. In this study the abnormal internal structure and the time when it appears have been established as occurring long before the abortions were recognisable externally. Second year aborted ovules described by Bramlett (1971) for P.echinata are much the same size as normal seeds and are more frequent than those of P.radiata (table 6:3). They are probably caused by other factors, one possibility suggested being insect damage.

### (3) Polyzygotic Polyembryony

In section 8 of the observations and results it was shown that clone 55 accommodated more pollen and more archegonia than clone 19. Although the clone 55 cone is larger, there are fewer ovules per cone, so the ovules are larger and have bigger micropyles and prothalli. This means that there is more space for pollen and archegonia. Sarvas (1962) noted that large P.sylvestris<sup>cones</sup> seemed to have more archegonia on average

than small ones, but the difference was not significant. Because of its size clone 55 has a smaller chance of ovule failure due to single pollen or single archegonia failing to develop. Buchholz (1948) considered that polyzygotic polyembryony had evolved as a mechanism for selecting the best of several available genotypes for the mature embryo. Ovules containing young embryos lying side by side, but reaching different depths in the corrosion cavity (photograph 44A), suggest that some embryos develop earlier or grow more vigorously in the early stages. Since fertilization in P. radiata appears to be simultaneous in eggs of one ovule and the physiological environment must be similar for each embryo, the differences in development must be due to differences in the genotype of the embryo. This idea has also been expressed by Sarvas (1962). However, it requires experimental study to prove that embryo competition does occur.

#### (4) The Application of Dissection and Microscopic Techniques to other Aspects of Seed Production

Data from the sampling of cones and direct counting of scales, ovules and aborted ovules can be used as a measure of the effect of controlled variables in an experimental situation. The effects of water regime, nutrient status and competition could be tested. Within clone variation between sites could be measured, so that sites where seed production factors are enhanced can be chosen for seed orchards.

The clonal numbers of archegonia can be recorded from microscopic sections. Using this information and the model given by Bramlett and Popham (1971), the germination percentages following self pollination experiments can supply information on the number of lethal genes occurring in each clone.

The most important consideration in this thesis has been clonal variation and its influence on seed production. Ideally, in setting up a seed orchard, clones should be selected, not only for their timber

yielding qualities, but also with a view to optimum seed production <sup>hec</sup> per <sup>are</sup> are. A suitable clone should carry a high number of cones per tree. Dissection counts of the number of viable ovules per cone and the seed germination percentages could also be used as criteria for the selection of clones.

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